

LOCAL-CIRCUIT MODULATION IN THE DENTATE
GYRUS INDUCED BY INCREASED NOREPINEPHRINE
FOLLOWING OREXIN-A INFUSION
INTO THE LOCUS COERULEUS

CENTRE FOR NEWFOUNDLAND STUDIES

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ROXANE SYNARD



Local-circuit Modulation in the Dentate Gyrus Induced by Increased Norepinephrine
Following Orexin-A Infusion into the Locus Coeruleus

by

Roxane Synard

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Local-circuit Modulation in the Dentate Gyrus Induced by Increased Norepinephrine Following Orexin-A Infusion into the Locus Coeruleus

The present thesis was designed to probe the local-circuit effects in the dentate gyrus following orexin-A infusion in or near the locus coeruleus as a manipulation to transiently increase norepinephrine levels. The study evaluated excitation and inhibition in the dentate gyrus as reflected in closely spaced pairs of perforant path evoked potentials in urethane-anesthetized rats. A paired pulse perforant path stimulation paradigm using three interstimulus intervals, in conjunction with a frequency dependent inhibition test, was used to probe local-circuit activity for early inhibition, facilitation and late inhibition. Measures of both the conditioning and test perforant path evoked potential excitatory post-synaptic potential (EPSP) slope and population spike amplitude were taken.

Orexin-A infusion, in or near the locus coeruleus, resulted in a gradually developing, significant enhancement of population spike amplitude and EPSP slope. Based a comparison of input-output data taken to prior to orexin-A infusion there were no differences in early GABA-A mediated inhibition or in midrange facilitation effects. The input-output data, however, supported an increase in late, GABA-B mediated inhibition following locus coeruleus activation. This was the first demonstration of a possible noradrenergic-induced enhancement of late paired pulse inhibition.

Frequency dependent inhibition results were disparate; interpreting the between group measures suggested that frequency dependent inhibition was reduced, whereas more sensitive within group measures were consistent with no change.

The increase of GABA-B mediated late inhibition initiated by norepinephrine may contribute to the ability of norepinephrine to reduce epileptic seizures and supports its role in promoting theta activity. Increased theta power has been implicated in learning and memory and would be consistent with norepinephrine's role in promoting plasticity.

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1. Introduction

The present thesis is concerned with modulation of local circuits in the dentate gyrus by the release of norepinephrine (NE) and in relation to long-term potentiation (LTP) triggered by NE. A reasonably large body of work exists describing the changes in the initial response to perforant path input produced by NE application or release in the dentate gyrus (e.g., Harley and Milway, 1986). However, relatively little work has been done examining NE's modulation of local-circuit effects, although in other brain areas NE has been reported to enhance GABAergic inhibition (Han et al., 2002), as well as glutamatergic actions.

A novel technique is used here to promote NE release, the infusion of the excitatory peptide orexin-A into the locus coeruleus. Orexin-A has previously been shown to tonically excite locus coeruleus neurons and to produce NE-dependent potentiation of the perforant path evoked potential for hours following infusion (Walling, 2004). Earlier results from our laboratory demonstrate that the increase in hippocampal NE produced by this method is transient and no longer evident 20 minutes after orexin infusion (Walling, 2004). Thus, both direct NE-dependent effects on local-circuit responses (within 20 minutes of infusion) and NE-LTP associated effects (at later time points) can be evaluated.

This introduction reviews the anatomy of the dentate gyrus, its advantages for electrophysiological studies, the anatomy and actions of the locus coeruleus-NE system and the paired pulse paradigms used here to probe local-circuit actions.

1.1 Anatomy of the Dentate Gyrus

The principle cells of the dentate gyrus are the granule cells. The dentate gyrus consists of three layers: the molecular layer (inner and outer), the cell layer, and the polymorphic region or hilus (Amaral and Witter, 1989).

The molecular layer consists of dendritic trees on which the perforant path and other terminals synapse. This layer is relatively cell free. The molecular layer is mainly occupied by the dendrites of granule, basket, and various polymorphic cells as well as terminal axonal arbors from several sources.

The granule cell layer is the most obvious in transverse section; it consists of densely packed columnar stacks of cell bodies, which form a U-shape or V-shape band depending on antero-posterior position. The arms of the U are termed the dorsal and ventral blades of the dentate gyrus. The granule cells send axons, the mossy fibers, into the hilus where they collateralize and then form synapses with the pyramidal cells of the CA3 region (Buhl et al., 1994). The granule cell has a characteristic cone-shaped tree of spiny dendrites with all of the branches directed towards the superficial portion of the molecular layer. Along the deep surface of the granule cell layer, basket cells with pyramidal shaped cell bodies are located between the granule cell and polymorphic layers. The majority of these cells are GABA-ergic and many are immunoreactive for other substances such as the calcium binding protein parvalbumin. Within the same region occupied by the basket cells are several other cell types with markedly different somal shapes and dendritic arrangements. Some of these are multipolar while others are more fusiform shaped. These cells have several aspiny dendrites entering the molecular

and polymorphic layers. It seems that all of these cells supply axons that contribute to the basket plexus in the granule cell layer. Many of these neurons are immunoreactive for GABA and are acting in large part as inhibitory interneurons (Buhl et al., 1994). Thus, there is a morphologically heterogeneous population of cells within, or slightly below, the granule cell layer that are interneurons and many contribute to the basket plexus surrounding the granule cells. However, there are also cells in this subgranular region that do not send their axons into the granule cell layer.

Between the dorsal and ventral blades is the polymorphic layer or hilus. The polymorphic cell layer is composed of a variety of neuronal cell types. The most common is the mossy cell. The cell bodies are large and are often triangular or multipolar in shape. Three or more thick dendrites originate from the cell body and extend for long distances within the hilus; often a dendrite will also extend through the granule cell layer and into the molecular layer. The mossy cell dendrites in effect never leave the confines of the polymorphic layer to enter the adjacent CA3 field. The most distinctive feature of the mossy cell is that all of the proximal dendrites are covered by very large and complex spines called “thorny excrescences” that are the sites of termination of the mossy fiber axons (Steward, 1976). The neurons in the hilus give rise to at least two intrinsic systems (Witter, 1993). One system, which originates in somatostatin and GABA-positive neurons, and apparently exerts an inhibitory effect, distributes to the outer two-thirds of the molecular layer. The other pathway, which arises from excitatory glutamatergic neurons, distributes preferentially to the inner one

third of the molecular layer and terminates densely at all other levels along the longitudinal axis of the dentate gyrus.

The interneurons form a local network in the dentate gyrus and function as neuromodulatory cells. Interneurons of the dentate gyrus may exert inhibitory influence upon their target cells (the granule cells) or they may inhibit other interneurons resulting in net excitatory influence on the target cells.

The granule cells give rise to distinct unmyelinated axons called mossy fibers. The mossy fiber terminals in the polymorphic layer establish contacts with the proximal dendrites of the mossy cells, the basal dendrites of the pyramidal basket cells, and other unidentified cells (Patton and McNaughton, 1995).

Overall, there appears to be at least three ways of providing inhibitory control over the excitatory granule cells (Amaral and Witter, 1995). There are a variety of basket cells located close to the granule cell layer, which contribute to the very dense terminal plexus that is restricted to the granule cell layer. The terminals in this basket cell plexus are GABA-ergic and form symmetric, presumably inhibitory contacts primarily on the cell bodies and shafts of apical dendrites of the granule cells. There is mounting evidence that GABA-ergic neurons in the polymorphic layer are themselves innervated by GABA-ergic terminals (e.g. Misgled and Frotscher, 1986). Given the extent a single basket cell plexus extends, it is conceivable that a single basket cell influences a very large number of granule cells. A second inhibitory input to granule cells originates from the axo-axonic or chandelier cells located in the molecular layer. These form symmetric contacts exclusively with the axons of granule cells.

There is a population of somatostatin immunopositive neurons scattered throughout the polymorphic layer. A dense plexus of immunoreactive fibers and terminals are also present in the outer two thirds of the molecular layer. The somatostatin cells located in the hilus, which colocalize with GABA, are the main source of this plexus of fibers and terminals in the molecular layer (Katona et al., 1999). This system of fibers, which forms contacts on the distal dendrites of the granule cells, provide a third means for inhibitory control of granule cell activity.

The inner third of the molecular layer receives projections that originate exclusively from cells in the polymorphic layer; this projection is called the associational/commissural projection (Amaral and Witter, 1995). The majority of terminals of this pathway form asymmetric, presumably excitatory, synaptic terminals on spines of the granule cell dendrites. Many of the axons contributing to these synaptic terminals appear to originate from the large mossy cells of the polymorphic layer. Because the mossy cells are immunoreactive for glutamate, it is likely that they release this excitatory transmitter at their terminals within the associational/commissural zone of the molecular layer (Patton and McNaughton, 1995). Associational fibers, in addition to contacting the spines of dentate granule cells, also contact the dendritic shafts of GABA-ergic basket cells. Therefore, the associational and commissural projections may operate as both a feed-forward excitatory pathway as well as a disynaptic feed-forward inhibitory pathway. It appears that the mossy fiber collaterals terminating on GABA/somatostatin cells in the polymorphic layer may lead predominantly to direct inhibition of granule cells at the

same septotemporal level and to either inhibition or excitation at more distant levels of the dentate gyrus.

The dentate gyrus is the “traditional” target of entorhinal-hippocampal fibers. In the molecular layer of the dentate gyrus, the perforant path terminals are confined to the outer two thirds as asymmetric synapses (Jones, 1993). Most of these synapses occur on the dendritic spines of granule cells, although a small portion of parvalbumin/GABA-positive neurons also synapse with perforant path fibers. Within the outer two thirds of the molecular layer, perforant path synapses make up at least 85% of the total synaptic population (Jones, 1993).

Fibers that originate in the lateral entorhinal area terminate in the outer one third of the molecular layer, and fibers from the medial entorhinal area terminate in the middle one third of the molecular layer (Abraham and McNaughton, 1984). The perforant path can be divided into three components: lateral, intermediate and medial. The intermediate pathway is thought to terminate in the molecular layer in between projections from the medial and lateral entorhinal areas. The lateral and medial components of the perforant path distribute their fibers differentially along the transverse axis of the dentate gyrus. The lateral perforant path projects preferentially to the suprapyramidal blade of the dentate gyrus, while the medial component either does not show a preference or predominantly targets the infrapyramidal blade. Lomo (1971) observed that with a stimulating electrode placed in the medial portion of the perforant path, close to the hippocampal fissure where the perforant path enters the dentate area, the incoming perforant path fibers divide the dentate area into a series of parallel segments. Andersen

(1971) emphasized that the positioning of the stimulating electrode was critical when recording in the dentate gyrus. When the perforant path was stimulated more laterocaudally, closer to the angular bundle, activation of the dentate gyrus was more widespread. The perforant path is believed to be glutamatergic, and therefore excitatory. There are no cells in the dentate gyrus that project back to the entorhinal cortex.

The hippocampus and dentate gyrus are known to have a remarkable capability for neuronal plasticity (Harley, 1991). When a stimulus is applied to the principle cells of the hippocampus two changes can occur: the cells are excited by the incoming impulse and if certain conditions are met permanent functional transformations can arise in the system of neurons. The permanent changes are referred to as plasticity. Chemical synapses have the capacity for short-term physiological changes, lasting seconds to a few hours, which increase or decrease the effectiveness of the synapse; this is referred to as short-term plasticity. Long term changes, lasting days or longer, can give rise to anatomical changes, including pruning of preexisting connections or even growth of new connections; this is referred to as long term plasticity. Anatomical alterations are typically long term and consist of the growth of new synaptic connections between neurons. The hippocampus displays evidence of both short and long term plasticity. When a new skill is learned or a new memory formed, something in the brain is changed in order to encode that memory. The hippocampus has been implicated as a target that is integral to the formation of new memories because groups of neurons in the hippocampus show plasticity of synaptic strength. Synaptic strength or efficacy refers to the effectiveness of presynaptic cell activity in inducing postsynaptic cell firing.

1.2 Measurement of Synaptic Efficacy

The regular and parallel arrangement of hippocampal neurons is an auspicious occurrence for the electrophysiologist, as it is possible to generate large extracellular responses via stimulation of excitatory afferents (Barnes, 1988). It is relatively easy to record extracellularly from a population of dentate gyrus granule cells because of the tightly packed nature of these cells. Technically, it is much easier to record a hippocampal field potential than to record the electrical activity of individual cells, moreover, the former provides a picture of how cell populations respond to stimuli.

After placing a stimulating electrode in the perforant path, a response in the millivolt range can be recorded in the dentate gyrus. This response is a measure of the excitability of a population of granule cells in the area of the recording electrode. The elicited response is referred to as an evoked field potential, or simply an evoked potential. In the dentate gyrus both the population EPSP and the population spike are revealed in the evoked potential resulting from perforant path stimulation.

The EPSP is a measure of the magnitude of synaptic efficacy; as stimulation is increased more transmitter is released and the magnitude of the EPSP increases accordingly. The EPSP is a measure of current flow into the dendrites of the postsynaptic cells in the area of the recording electrode. Factors that influence the EPSP are the number of perforant path fibers activated by stimulation, the amount of neurotransmitter released, and the sensitivity and number of postsynaptic receptors (Lomo, 1971). The placement of the recording electrode within the dentate gyrus influences the resulting response. If a recording is made from the molecular layer (dendrites) the EPSP is a

negative going wave reflecting an inward current flow. Further downward movement of the recording electrode places it in the granule cell layer (cell bodies), and the evoked potential reverses, which reflects an outward current flow as a positive going wave.

When the magnitude of the EPSP passes threshold, granule cell action potentials occur. The population spike, the other constituent of the evoked potential, is a measure of the number of postsynaptic cells that fire. The synchrony of granule cell firing near granule cell bodies appears as a negative deflection overlying the positive going EPSP.

1.3 Modulation of Hippocampal Neuronal Transmission

Neuronal excitability and transmission through the hippocampal formation are modulated by sensory input and behavioral state. For example, Herreras et al. (1988) found that the dentate gyrus responses to perforant path stimulation were enhanced when the animal had its fur stroked. Cellular excitability was also increased as judged by a left shifting of the EPSP-population spike relationship. Responses within area CA1 exhibited quite different reactions. Sensory stimulation produced a dramatic decrease in both EPSP and population spike amplitude. The decrease in EPSP induced by sensory stimulation completely accounted for the reduction in population spike amplitude, as indicated by a lack of variation in EPSP-population spike relationship. Results demonstrate that sensory stimulation causes a modification of information transfer through the hippocampus. The alteration of hippocampal transmission can serve to gate the information reaching CA1 and dentate gyrus.

Various types of anesthesia are known to affect evoked potentials in the dentate gyrus. For example, Shirasaka and Wasterlain (1995) examined the effects of urethane

anesthesia. Urethane is a commonly used anesthetic in physiological and pharmacological studies of animal central nervous system. Urethane has been noted to decrease several measures of central nervous system activity, including decreased spontaneous firing rates in the hippocampus (Mercer et al., 1978). Using an input-output paradigm Shirasaka and Wasterlain demonstrated that at lower stimulus intensities population spike amplitude and EPSP slope were significantly lower in urethane-anesthetized animals compared to awake animals; these differences disappeared at higher stimulus intensities. It seems that urethane is either suppressing excitatory neuronal activity in dentate granule cells pre- or post-synaptically, or urethane is directly or indirectly affecting the GABAergic inhibitory system.

Maroun and Richter-Levin (2002) have illustrated an age-related change in local-circuit plasticity within dentate gyrus granule cells. Using several different methods they were able to demonstrate an age-related impairment in both population spike amplitude and EPSP slope: young animals (4-8 months) had significantly higher measures than old animals (22-28 months). However, population spike/EPSP ratios did not differ between groups, indicating there was no difference in the excitability of dentate granule cells. Using frequency dependent inhibition as a measure of local-circuit plasticity they showed that aged rats did not exhibit any reduction in frequency dependent inhibition following theta burst stimulation while young rats did. Both group showed similar long-term potentiation to theta burst stimulation. Based on this outcome they suggest changes in local-circuit plasticity, as reflected in frequency dependent inhibition, may be important for memory processes that are impaired in aged rats.

1.4 The Locus Coeruleus is a Source of Norepinephrine

The major supply of NE to the hippocampus, as well as to other brain areas, is the nucleus located in the pons known as the locus coeruleus (Loy et al., 1980). The locus coeruleus is a small dense group of NE containing cell bodies whose projections account for the majority of NE input to the hippocampal formation. Locus coeruleus axons reach the hippocampus via three distinct routes: the ventral amygdaloid pathway, the fornix, and the cingular bundle (Loy et al., 1980). Fibers from these routes innervate the dentate gyrus in a distinct dorsal to ventral pattern. Fibers from the locus coeruleus form the dorsal noradrenergic bundle before linking with the medial forebrain bundle and then entering the hippocampus. The hilar region of the dentate gyrus receives the densest projections from the locus coeruleus; however, there are less dense projections to the molecular and granule cells layers. The highly clustered appearance of locus coeruleus neurons reinforces the idea that these cells would be activated in unison and the output would be relatively synchronous.

Harley and Milway (1986) demonstrated that glutamate injected into the locus coeruleus induced a significant increase in amplitude of the perforant path-evoked population spike in the dentate gyrus. It was suggested that glutamate injection into the locus coeruleus specifically mimicked normal physiological locus coeruleus activation, increasing the release of NE in the dentate gyrus. Further support for this is illustrated in Harley's previous studies (e.g., Neuman and Harley, 1983), which demonstrated that NE, iontophoresed in the dentate gyrus at the cell body layer, produced similar increases in the population spike amplitude.

In the awake animal the level of locus coeruleus firing is a direct function of arousal. Cells fire the slowest during sleep and increase to higher levels with alert arousal (Aston-Jones and Bloom, 1981), which is likely when an animal would learn or form a memory. Sara et al. (1994) used recordings of single unit activity in the locus coeruleus during learning, during spontaneous exploration of a novel environment and unfamiliar objects and during the presentation of auditory stimuli to illustrate that locus coeruleus neurons respond to novel stimuli, habituate rapidly, and respond again when the stimulus changes its predictive value, well before noticeable responding at a behavioral level. These results provide support for the suggestion that the noradrenergic system is implicated in shifting attention to vitally important environmental conditions. In awake animals, any behaviorally arousing stimulus results in a phasic increase of firing in the locus coeruleus. The firing rate of NE cells is highly dependent upon behavioral state, sensory input from all modalities and changes of significance of environmental stimuli. Aston-Jones and Bloom (1981), through a series of experiments, were able to demonstrate that, although NE positive locus coeruleus neurons discharge in a slow tonic manner overall, mean rates change significantly as a function of the naturally occurring sleep wake cycle. Tonic discharges were highest during waking, slower during slow-wave sleep, and were nearly absent preceding and throughout paradoxical sleep. Aston-Jones and Bloom were also able to demonstrate fluctuation in locus coeruleus NE activity during certain waking behaviors. For example, during grooming or consuming sweet water discharges were decreased, while the locus coeruleus exhibited bursts of discharges during orienting

responses to stimuli. This again is consistent with the role of the locus coeruleus as an attention shifter.

1.5 Exogenous and Endogenous Norepinephrine and Neuromodulatory Actions

1.5.1 Exogenous Norepinephrine

Initially, NE was classified as an inhibitory neurotransmitter. Segal and Bloom (1976) observed generalized inhibitory responses in hippocampal neurons in response to noradrenergic stimulation and showed that activation of the locus coeruleus induced similar responses. However, others have provided evidence for a facilitatory role of NE in that it enhances the signal to noise ratio in the neocortex and cerebellum by suppressing spontaneous activity of neurons and enhancing the activity evoked by sensory stimuli (e.g., Hasselmo, 1995). Both iontophoretic application of NE (Neuman and Harley, 1983) and locus coeruleus stimulation (Harley et al., 1989) have been shown to potentiate population spike amplitude within the dentate gyrus. A neuromodulatory effect of NE has been reported in the limbic system (see Harley, 1987 for review), which suggests that NE is involved in modulation of arousal and stimulation.

In an *in vitro* experiment conducted by Lacaille and Harley (1985), superfusion of 10 μ M NE enhanced population spike amplitude and EPSP slope, and decreased onset latency of the population spike. These results are similar to other *in vitro* studies (Stanton and Sarvey, 1987). However, the effect of NE on EPSP slope could not account for the increased population spike amplitude also produced by NE in the same slice. This indicates that NE produces an increase in cell responsiveness to synaptic inputs.

In contrast, Neuman and Harley (1983) did not observe a consistent change in EPSP slope, which is typically the case *in vivo*. Using iontophoretically applied NE a long lasting potentiation of the granule cell population spike was observed. It seems that NE alters the coupling between EPSP and population spike generation, which brings a larger number of cells to threshold rather than simply potentiating excitatory synaptic drive. Although not specifically altering synaptic strength, these results suggest NE does induce long-term changes in hippocampal responsivity.

1.5.2 Endogenous Release of NE

Endogenous release of NE from locus coeruleus projections in the hippocampus can also induce potentiation of the perforant path evoked potential in the dentate gyrus. Stimulation of locus coeruleus neurons results in endogenous release of NE.

An enhancement in perforant path induced population spike amplitude and a reduction in population spike onset latency can be produced by several methods of activating locus coeruleus neurons, including electrical stimulation, injection of glutamate, orexin-A or normal saline (e.g., Harley et al., 1989; Harley and Evans, 1988). Transient increases in population spike amplitude can also be produced by mechanical stimulation of the locus coeruleus (personal observation).

Dahl and Winson (1985) varied perforant path and locus coeruleus stimulation while recording in the dentate gyrus. Locus coeruleus stimulation prior to perforant path stimulation resulted in a decrease in EPSP slope, an amplification of population spike amplitude and a decrease in onset latency of the population spike. These changes were recorded at the granule cell layer, while no changes were detected at the molecular layer.

Thus, the effects of locus coeruleus stimulation on EPSP may depend on the recording electrode position in the dentate gyrus.

Harley et al. (1989) electrically stimulated the locus coeruleus while stimulating either the ipsilateral or contralateral perforant path. A pipette electrode assembly was also used to inject glutamate into the locus coeruleus. During ipsilateral perforant path stimulation an increase in population spike amplitude was seen following the offset of electrical locus coeruleus stimulation. The effects on EPSP slope and population spike onset latency were variable. Pairing locus coeruleus stimulation with contralateral perforant path activation also potentiated the population spike amplitude as measured in the contralateral dentate gyrus during locus coeruleus stimulation, albeit to a lesser extent than ipsilateral stimulation. It should be noted that propranolol, a beta-adrenoceptor blocker, had no effect on population spike potentiation produced by electrical stimulation of the locus coeruleus, but blocked the potentiation associated with glutamate stimulation. It appears that there is a separate system that can be activated in the vicinity of the locus coeruleus, which is capable of potentiating evoked potentials in the dentate gyrus, but is not dependent on beta-receptor activation. Both short and long-term potentiation produced by exogenous NE and by glutamate activation of the locus coeruleus have been shown to be critically dependent on the activation of beta-receptors (Harley et al., 1989).

Harley and Evans (1988) reported that glutamate injections into the locus coeruleus produced a significant enhancement of the population spike that occurred within 90 seconds of injection. EPSP slope was significantly increased, although the effects were

typically briefer than effects on population spike amplitude. Population spike onset latency, in contrast, was generally unaffected by locus coeruleus activation. Injections of saline into the dentate gyrus had no effect on population spike, EPSP slope or onset latency. However, injections of timolol or propranolol into the dentate gyrus prior to glutamate injections into the locus coeruleus blocked the potentiation, and decreased population spike amplitude in response to locus coeruleus activation. Thus, most of the potentiating effects of NE in the dentate gyrus appear to be mediated by beta-adrenoceptors, while alpha-adrenoceptors may mediate decreased responsivity.

More recently the orexins have been used to stimulate the locus coeruleus. Hagan et al. (1999) revealed an exceptionally dense innervation of orexin-A immunoreactive fibers and varicosities in extrahypothalamic areas, particularly the locus coeruleus. Furthermore, concentrations of orexin-A ranging from 30 nM to 3 μ M were shown to increase the rate of firing of locus coeruleus neurons. Orexin-A induced enhancement of locus coeruleus neuronal firing highlights one route for involvement of the peptide in maintaining the waking state, as cells in this area are maximally active during arousal but are virtually dormant during rapid eye movement sleep (Aston-Jones and Bloom, 1981). Both orexin-A and -B are believed to play a broad regulatory role in the central nervous system and possibly regulate arousal, hormonal control, appetite and other functions (Hagan et al., 1999). Horvath et al. (1999) demonstrated that hypocretin (orexin-A) provides a large synaptic input to both the rodent and primate noradrenergic neurons in the locus coeruleus.

1.6 Paired Pulse Paradigm

Evoking two perforant path potentials in rapid succession reveals a change in the response to the second pulse (P2) relative to the response to the first pulse (P1). P1 is referred to as the conditioning pulse and P2 as the test pulse (Steward, 1976). The conditioning pulse allows for the measurement of the excitatory response of the granule cells, while concurrently activating the local hippocampal interneuronal network. Observing the test pulse allows for the examination of the modulatory effect the local network has on the excitatory response. The differences in the resultant response are dependent on the interval between conditioning and test pulses, called the interstimulus interval (DiScenna and Teyler, 1994).

With an interstimulus interval of 10–40 msec, P2 is smaller than P1; this is referred to as early onset paired pulse inhibition (Burdette and Gilbert, 1995). The second evoked response is strongly inhibited when stimulation of the perforant path is spaced at this interval. The second response is less strongly inhibited with interstimulus intervals of 300–1000 msec; this is referred to as late onset paired pulse inhibition (Blaise and Bronzino, 2000). Both types of paired pulse inhibition are believed to be mediated by both feed-forward and feedback mechanisms (Sloviter, 1991). DiScenna and Teyler (1994) were able to show that early inhibition is GABA_A-mediated while late inhibition is GABA_B-mediated.

At interstimulus intervals of 40–200 msec P2 is larger than P1. This is called paired pulse facilitation and is thought to result from a selective increase in NMDA-mediated

synaptic response to the second stimulation (Joy and Albertson, 1993). Paired pulse facilitation may also influence feed-forward inhibition.

The level of inhibition or facilitation produced with these interstimulus intervals varies depending on whether the medial or lateral perforant path is being stimulated. According to the results of Rich-Bennett et al. (1993) the levels of early and late inhibition recorded are lower when stimulating the medial perforant path, whereas the level of facilitation is increased, when compared to levels of inhibition or facilitation recorded while stimulating the lateral perforant path. These effects are intensified with the use of GABA_A antagonists or GABA_B agonists.

DiScenna and Teyler (1994) set out to determine the stage of growth at which inhibition and facilitation develop. They found that by postnatal day 6, the day at which a population spike could be evoked, paired-pulse effects on granule cell discharge indicated the presence of weak early onset inhibition. By postnatal days 7/8 the level of inhibition was equal to that of a mature animal. Late onset inhibition was equivalent to a mature animal by postnatal day 6. The most consistent difference between the mature and developing dentate gyrus occurred at interstimulus intervals that should produce population spike facilitation, where it was significantly depressed in developmental groups, until after the third postnatal week when was it equal to mature animals. This study indicated that short-term plasticity matures rapidly in the dentate gyrus and that the inhibitory circuitry can function at a very early age.

Blaise and Bronzino (2000) also examined the development of the paired pulse response. They found significantly less early and late inhibition and facilitation in

developing animals compared to mature animals. However, a major difference in methodology might explain these differences, as Blaise and Bronzino utilized *in vivo* procedures, while DiScenna and Teyler utilized *in vitro* procedures. The differences may reflect the consequences of having extra-hippocampal connections removed in *in vitro* preparations. The findings of Blaise and Bronzino are most likely due to modulation of local-circuit inhibitory interneuronal activity by extra-hippocampal inputs such as the noradrenergic projections from the locus coeruleus. The functionality of systems responsible for modulation of dentate granule cell excitability appears to depend on age.

Blaise and Bronzino also examined vigilance and its effects on paired pulse responses. While differences in the paired pulse index did occur during quiet waking, slow-wave sleep, and REM sleep, there was no significant effect of vigilance state on early inhibition, facilitation or late inhibition.

1.7 Frequency Dependent Inhibition

Frequency dependent inhibition is an additional method of examining local-circuit modulation within the dentate gyrus. Sloviter (1991) was one of the first to examine frequency dependent inhibition as a method of evaluating feed-forward and feedback inhibition in the hippocampus. With frequency stimulation at 0.1 Hz, paired pulses delivered 40 msec apart evoke no inhibition of the spike despite the fact that the first granule cell population spike is large. Recurrent inhibition is evident at an interstimulus interval of 20 msec, but it is weak. With frequency stimulation delivered at 1.0 Hz, the first spike paradoxically decreases in amplitude; this is frequency dependent first spike inhibition. Despite this decrease in first spike amplitude, inhibition of the second spike is

increased, that is the second spike is smaller at 1.0 Hz than at 0.1 Hz. The relatively weak inhibition evoked by stimulus pairs delivered at low frequencies is primarily recurrent inhibition, which is defined as the inhibition initiated by granule cell discharges. As afferent stimulus frequency is increased from 0.1 Hz to 1.0 Hz, both feed forward inhibition and the effectiveness of paired pulse inhibition is increased, while the amplitude of the first spike decreases.

Rosenblum et al. (1999) took Sloviter's ground breaking work one step further and examined frequency dependent inhibition within the dentate gyrus by increasing perforant path stimulation from 0.1 Hz to 1.0 Hz for a short interval. Sloviter (1991) was able to demonstrate that frequency dependent inhibition is GABA-mediated and reflects mainly feed-forward inhibition by perforant path activated interneurons contacting dentate gyrus granule cells. Using the modified frequency dependent inhibition procedure, Rosenblum et al. (1999) revealed that frequency dependent inhibition in the dentate gyrus is NMDA dependent. After application of MK-801, an NMDA blocker, the level of population spike amplitude inhibition was dramatically reduced; in contrast, the NMDA antagonist did not affect the frequency dependent inhibition of the EPSP slope. The results of this study suggest that NMDA receptor mediated currents contribute to the basal level responses of inhibitory interneurons. Frequency dependent inhibition requires activity of NMDA receptors.

Furthermore, Maroun and Richter-Levin (2002) were able to demonstrate that frequency dependent inhibition is compromised in aged animals. This reduction in frequency dependent inhibition may be a reflection of a loss of local-circuit plasticity in

aged animals. Aging is associated with memory impairments and with a loss of interneurons. The age-related impairment in local-circuit plasticity may be the consequence of the loss of interneurons.

1.8 The Present Study

The purpose of the current study was to study the modulatory effects of NE in the dentate gyrus of the hippocampus. The specific goals were to:

- 1.) investigate potentiation of the dentate gyrus population spike with the infusion of orexin-A into the locus coeruleus;
- 2.) examine the paired pulse paradigm within dentate gyrus granule cell circuitry;
- 3.) utilize frequency dependent inhibition as a method of examining local-circuit plasticity within the dentate gyrus;
- 4.) assess the effects of locus coeruleus activation on paired pulse responses and on measures of local-circuit plasticity in the dentate gyrus.

The study evaluated the effects of NE on excitation and inhibition of the perforant path evoked potential by using the administration of orexin-A to increase NE levels in the hippocampus and by using the paired pulse paradigm and frequency dependent inhibition to probe local-circuit activity.

2. Methods

2.1 Subjects

Subjects were 12 male Sprague-Dawley rats (Memorial University Vivarium) weighing 250-350g. Animals were provided food and water ad lib. The experimental protocol conformed to the IACC/CCAC guidelines. Each animal was anesthetized with urethane (1.5g/kg i.p.) and placed skull-flat in a stereotaxic frame. Supplemental doses of urethane (10% initial dose) were given at 30-minute intervals following the first injection if animals continued to respond to foot pinch. No animal required more than 2 supplements. Core body temperature was maintained at $37\pm 1^{\circ}\text{C}$ using a rectal probe coupled to a heating pad and temperature control unit. Small holes were drilled in the skull at points referenced from bregma to accommodate electrodes in the dentate gyrus and perforant pathway, and a cannula in the locus coeruleus.

2.2 Orexin-A Preparation

Orexin-A was prepared to concentrations of 1, 10 and 100 nM in 0.9% sterile saline. Initially, 500 μL of orexin-A was stored in polypropylene containers; following determination of a storage container problem the peptide was stored in glass autosampler vials. The prepared orexin-A was stored in a -20°C freezer. The peptide was used within eight weeks of preparation.

2.3 Electrode Placements

A bipolar stimulating electrode (Rhodes NE 100) was placed in the perforant path of the left hemisphere (7.2 mm posterior, 4.0 mm lateral from bregma). A stainless steel unipolar electrode was directed at the cell body layer or the hilus of the dentate gyrus (3.5

mm posterior and 2.0 mm lateral from bregma). The depth of the electrode was optimized by monitoring the population spike recorded from the dentate gyrus as a response to a single square wave monophasic stimulus delivered through the perforant path. The recording electrode was positioned to attain a maximum size population spike; optimal depths ranged between 2.5 and 3.5 mm. The optimized population spike size ranged between 3 and 6 mV. An outer guide cannula (22 gauge) was directed at the LC at a 20° angle (12.6 mm posterior and 1.3 lateral to bregma and 3.6 mm ventral from skull surface); an inner cannula (28 gauge) extended an additional 2.4 mm beyond the guide cannula.

2.4 Stimulation and Recording of Evoked Potentials

The 'Workbench' program in the commercial electrophysiology software package 'Brainwave' (Datawave Technologies) was used to control the stimulation and recording parameters in the experiment. Two monophasic 0.2 milliseconds pulses with interstimulus intervals of 20, 100 and 500 milliseconds, to generate paired pulse early inhibition, facilitation, and late inhibition, respectively, were generated by a Datawave A/D board and delivered through a constant current unit at a frequency of 0.1 Hz. Two samples of each paired pulse measure were thus taken every minute. Signals were amplified 50x–200x using a bandpass setting of 0.1–3.0 KHz by a Grass preamplifier and displayed on a dual channel digital oscilloscope. Waveforms were digitized on-line at a rate of 10,000 points/second, displayed on a computer monitor and stored using Brainwave and a personal computer.

2.5 Experimental Protocol

For each subject stimulating electrodes were lowered first. Then, animals received single pulse stimulation typically at 400-600 μA current as recording electrodes were lowered to approximate depths of 2.5-3.5 mm below brain surface until maximal negative-going population spikes with latencies of 5 milliseconds or less were obtained.

First, a baseline measure was collected using a single pulse stimulation of 400-600 μA .

Second, an input-output curve was obtained using interleaved paired pulses (with interstimulus intervals to induce early inhibition, facilitation, and late inhibition), beginning at a current of 16 μA and then proceeding on a scale to 1260 μA (16, 32, 64, 100, 250, 500, 750, 1000, and 1260 μA). Three interstimulus intervals were executed one after another with a 10 sec delay between each paired pulse. Every 30 seconds current was increased until the series was completed from 16-1260 μA . This procedure was repeated until 6 complete input-output sets were completed.

In the third step, stimulation was adjusted to achieve a 50% population spike on P1. A frequency dependent inhibition probe was then employed. This consisted of ten pulses at 0.1 Hz followed by ten pulses at 1.0 Hz; this was repeated twice.

In the fourth step, using the previously obtained 50% population spike current a 30 minute baseline period was recorded using the three interleaved paired pulses.

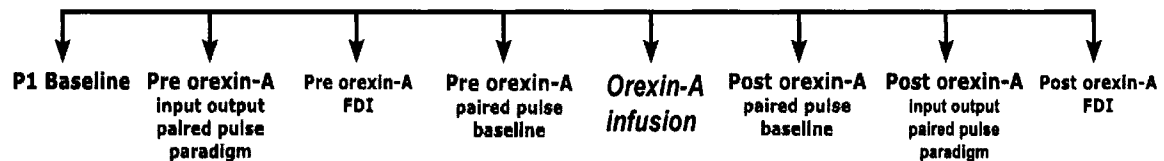
The fifth step was at the end of the baseline period. Using a Hamilton syringe, 200 nl of a 1 nM or a 100 nM solution of orexin-A (California Peptide) was injected into the locus coeruleus for a two minute period.

The interleaved paired pulses were continued for an additional 30 minutes as the sixth step.

The seventh step included a post-orexin-A input-output curve that used the same current values and time intervals as used in the pre-drug input-output curve.

In the final and eighth step, the frequency dependent inhibition probe was repeated. This was followed by frequency dependent inhibition probes with the current reduced to match the population spike to its pre-orexin-A amplitude.

The experimental protocol is illustrated below.



2.6 Histology

Immediately after the recording procedure the subject was removed from the stereotaxic apparatus and decapitated with a guillotine. Within 10 minutes of sacrifice, the brain was removed and frozen in approximately 20 mL of 2-methyl butane that had been previously cooled in a -80°C freezer for at least 20 minutes. Brains were stored in the same freezer until sectioning.

For verification of electrode and cannula placement, brains were cut into $40\ \mu\text{m}$ sections on a Jung Frigocut cryostat microtome and placed on glass microscope slides. Sections were then subjected to an acetylcholinesterase metachromatic Nissl staining procedure. During sectioning alternate slices were subjected to active and total glycogen

phosphorylase staining procedure to facilitate localization of the stainless steel recording electrode. Slides were then analyzed using a projecting microscope.

2.7 Evoked Potential Parameter Extraction

For each animal the parameter extraction option in the 'Workbench' program was used to measure the population spike amplitude and EPSP slope. Measurement #25 (Peak to Valley) from the 'Electrophysiology' function set was used to obtain population spike amplitude while measurement #1 was used to determine EPSP slope.

2.8 Statistical Analysis

Five minute averages were analyzed using a repeated measures ANOVA to compare pre vs. post orexin-A population spike and EPSP slope for P1, P2, and P2/P1 ratios. A post hoc Duncan multiple range analysis with $p < 0.05$ was performed when the repeated measures ANOVA proved significant.

Using the input-output curve data, sets with similar pre and post P1 values were selected to match the P1 measures and test the change in paired pulse ratios when P1 was matched. P2/P1 ratios for each of the paired pulses were averaged to get an average ratio for each condition with a matched P1 value. Repeated measures t-tests were then performed on the P2/P1 ratio values (e.g., pre P1 500 μ amp vs. post P1 250 μ amp) to obtain measures of facilitation and inhibition.

Correlations between EPSP slope and population spike were examined pre and post orexin-A. Correlations between the paired pulse ratios and their associated P1 population spikes and EPSP slopes were also examined. For completeness, a correlational analysis

of P1 population spike amplitude to P2/P1 EPSP slope for each of the paired pulse measures was conducted.

For frequency dependent inhibition, the population spike amplitude or EPSP slope of responses 2-4 at 0.1 Hz was averaged and compared with that of the averaged last 3 responses at 1.0 Hz in each set. The results of the 2 sets were averaged. This procedure follows that of Rosenblum et al. (1999). Paired t-tests were used to compare percent inhibition produced by frequency dependent inhibition pre and post orexin-A.

3. Results

3.1 General

Two of the 12 animals were excluded from the analysis because of unexplained changes in the evoked potential parameters. The remaining 10 animals were divided into two groups. The experimental group consisted of four animals that had significant enhancement of P1 population spike amplitude characteristic of noradrenergic modulation in the dentate gyrus. All of these animals had injection tip placements in or within 200 microns of the locus coeruleus (Figure 1a). Orexin-A for these animals was also stored in appropriate glass containers. The experimental animals received a 100 nM concentration of orexin-A. The control group consisted of 6 animals that did not have any significant increase in P1 population spike amplitude post-injection; the sixth step as outlined in the methods section. Two had injection sites outside of the locus coeruleus (Figure 1b), these animals received a 100 nM concentration of orexin-A; while four had accurate site placement but were assumed to have inadequate levels of orexin due to storage of the peptide in polypropylene to which it adheres (Kastin and Akerstrom, 1999). Two of these animals received a 1 nM concentration of orexin-A (11C and 12 C), while the other two animals received a 100 nM concentration of orexin-A (13C and 14C). Previous work showed that both 1 and 100 nM concentrations of orexin-A produced NE induced potentiation of the population spike in the dentate gyrus. The orexin-A given to the control animals with accurate site placement in the locus coeruleus, perforant path and dentate gyrus was assumed to be inactivate because no population spike potentiation was observed. In addition, several other researchers in the laboratory using orexin-A

stored in polypropylene containers did not observe population spike potentiation when accurate site placement was used. When orexin-A was stored in the appropriate glass autosampler vials population spike potentiation was observed when accurate electrode and injection tip site placements were employed.

3.2 Effect of Orexin-A on the Response to the First Pulse (P1)

3.2.1 Experimental Group

Mean baseline population spike amplitude for the experimental group (n=4) was 3.06 mV (± 1.43); mean post orexin-A population spike amplitude was 3.73 mV (± 1.77) for an average increase to 122% of baseline. Mean baseline EPSP slope for the experimental group was 4.19 mV/ms (± 1.62); mean post orexin-A EPSP slope was 4.30 mV/ms (± 1.69). Here the average increase was 103% of baseline.

Figure 2 illustrates the P1 normalized population spike amplitude for the experimental group; P1 potentials following orexin-A infusion were normalized to P1 potentials following orexin-A infusion. The repeated measures ANOVA revealed that P1 population spike amplitude significantly increased over time ($F_{11,33}=9.58$; $p<0.05$). Duncan post-hoc comparisons at $p=0.05$ showed that P1 population spike amplitude increased at the point of orexin-A injection and continued to increase (up to 26% over baseline) over the 30 minutes post injection period for the experimental group.

Figure 3 shows the P1 normalized EPSP slope for the experimental group. The repeated measures ANOVA revealed that P1 EPSP slope also significantly increased over the baseline period ($F_{11,33}=2.64$; $p<0.05$). Duncan post-hoc comparisons at $p=0.05$ showed that P1 EPSP slope was significantly greater than baseline at 10 minutes post

orexin-A and consistently larger from 20 minutes out, until the end of the post injection period with a final percentage increase of 8%.

A correlational analysis was conducted to determine the relationship between P1 EPSP slope and P1 population spike amplitude. Table 1 shows the results of this analysis for the experimental group. During the baseline period for two of four animals there was no correlation between EPSP slope and population spike amplitude; post orexin-A there was a significant negative correlation suggesting increased cell excitability. Larger population spikes occurred with smaller EPSPs. For the other two experimental animals there was a significant positive correlation between population spike amplitude and EPSP slope both during the baseline and post orexin-A injection. For these animals the correlations increased once the norepinephrine system was activated by orexin-A.

3.2.2 Control Group

As noted there were no differences pre and post injection in population spike amplitude for the control group. Mean baseline population spike amplitude for this group was 2.58 mV (± 0.37); post injection population spike amplitude was 2.53 mV (± 0.38). The control group EPSP slope also did not differ pre and post injection. Mean baseline EPSP slope for the control group was 3.27 mV/ms (± 0.36); mean post injection EPSP slope was 3.20 mV/ms (± 0.38).

Figure 2 illustrates the P1 normalized population spike amplitude for the control group.

Table 2 illustrates the results of the EPSP slope – population spike amplitude correlational analysis for the control group. No strong pattern or relationship between

these two measures was seen for this condition. During the baseline period three of 6 control animals showed a positive correlation between EPSP slope and population spike amplitude; after the orexin-A injection only one of the 6 animals maintained this positive correlation.

3.3 Effect of Orexin-A on the Response to the Second Pulse (P2)

3.3.1 Effect of Orexin-A on Experimental Group P2 Potential

A repeated measures ANOVA was performed on P2 potentials to determine whether the effects of orexin-A are similar to the effects on P1 potentials. This analysis was conducted with the P2's for each of the three respective paired pulse conditions. For the early inhibition paired pulse for the experimental group, the repeated measures ANOVA revealed the P2 population spike amplitude tended to decrease post orexin-A compared to baseline ($F_{11,33}=2.06$; $p<0.05$). The P2 population spike decreased over the 30 minute post injection period. The facilitation paired pulse P2 population spike amplitude tended to increase, but this was not significant ($F_{11,33}=1.85$; ns). Finally, the P2 associated with late inhibition showed a significant increase ($F_{11,33}=2.56$; $p<0.05$). Duncan post-hoc comparisons at $p=0.05$ showed that P2 population spike amplitude increased at the point of injection and continued to increase until the end of the post injection period with a final percentage increase of approximately 22%, a similar pattern to that of P1 overall. It appears then that orexin-A increased the P2 population spike outside of the early inhibition window.

Repeated measures ANOVA were also conducted on P2 EPSP slope for each of the paired pulses. None of these individual analyses were significant for the experimental

group. However, across all of the paired pulse conditions, there was a trend for P2 EPSP slope to increase over the 30 minute post orexin-A injection period as did P1 EPSP slope ($F_{11,33}=1.56$; ns).

3.3.2 Effect of Orexin-A on Control Group P2 Potential

Repeated measures ANOVA conducted on P2 population spike amplitude for the control group revealed no significant difference pre and post orexin-A for the early inhibition P2 or the facilitation P2 potentials ($F_{11,33}=0.30$, ns; and $F_{11,33}=1.84$, ns, respectively). The late inhibition P2 decreased significantly in the control group but the decrease appeared to be an anomaly as it began 15 minutes prior to injection ($F_{11,33}=3.82$; $p<0.05$).

For the control group, the repeated measures ANOVA for P2 EPSP slope associated with early inhibition ($F_{11,33}=2.21$; $p<0.05$) and facilitation ($F_{11,33}=3.27$; $p<0.05$) revealed a decrease over time. The P2 EPSP slope associated with late inhibition did not change. The repeated measures ANOVA for late inhibition was not significant for P2 EPSP slope ($F_{11,33}=1.12$; ns). It seems that in the control group, as with P2 population spike amplitude, P2 EPSP slope decreased over time without any experimental manipulation.

3.4 Effect of Orexin-A on Input-output Relations

3.4.1 Experimental Group

Figure 4 illustrates the change in P1 population spike amplitude over the range of input-output current values for the experimental group. At all currents with a measurable population spike, population spike amplitudes are larger following orexin-A activation of the norepinephrine system than before this activation. Figure 5 presents the variation in

P1 EPSP slope over the extent of input-output current values for the experimental group. Again, the P1 EPSP slopes are larger following the orexin-A injection; however, they are not of the magnitude of the increase in P1 population spike.

Based on Figure 4, P1 population spikes were selected for the experimental group that had similar amplitudes prior to and following orexin-A injections. This required using population spikes at different currents. P2/P1 ratios were then compared at these current levels. Current values that were selected were 500 μ amp pre injection and 250 μ amp post injection. The mean P1 population spike amplitude at 500 μ amp prior to injection was 1.94 mV (± 0.19); the mean P1 population spike amplitude at 250 μ amp following the injection was 1.49 mV (± 0.33). The difference between these means was not significant ($t_3=2.42$, ns).

3.4.2 Control Group

Control group input-output curves for population spike and EPSP slope did not change (Figures 8 and 9). Since current changes were not needed to match population spikes, for the parallel analyses 500 μ amp was chosen to compare pre - post values. The mean P1 population spike amplitude at 500 μ amp prior to injection was 1.63 mV (± 0.074); the mean P1 population spike amplitude at 500 μ amp following the injection was 1.61 mV (± 0.095). As expected the difference between these means was not significant ($t_5=0.22$, ns).

3.4.3 Effect of Orexin-A on Paired Pulse Index

Significant negative P1 to P2/P1 correlations ($p<0.01$), ranging from -0.54 to

-0.95 across the four experimental subjects for all paired pulse conditions, suggest that larger P1s are associated with smaller P2s even at intervals where inhibition is not predicted (i.e., facilitation) and justify the use of the P1-matching technique in the analysis of paired pulse measures, described in the Methods section. All population spike correlations for the experimental and control groups can be found in Tables 3 and 4.

3.4.3.1 Effect of Orexin-A on Experimental Group Paired Pulse Early Inhibition

The mean early inhibition of the population spike amplitude paired pulse ratio for baseline was 0.28 (± 0.13) for the experimental group. Post orexin-A the early inhibition population spike amplitude paired pulse ratio was reduced to 0.23 (± 0.10). The repeated measures ANOVA revealed that P2/P1 was significantly smaller post orexin-A than during the baseline period ($F_{11,33}=3.84$; $p<0.05$). Duncan post-hoc comparisons at $p=0.05$ showed that early inhibition P2/P1 population spike amplitude ratio was significantly smaller at 15 min post orexin-A and continued to decrease to approximately 0.19 at the end of the 30 min record (Figure 6). EPSP slope ratios during baseline and post orexin-A did not differ (Table 5).

Table 3 presents the P1 vs. early inhibition P2/P1 correlation analysis for population spike for the experimental group. Prior to orexin-A, for two animals the P1 vs. P2/P1 for early inhibition population spike amplitude correlation was non-significant; the correlation for the other two animals during this period was negative and significant at $p=0.01$. For the period following orexin-A activation of the norepinephrine system correlations for all animals were negative and significant; this analysis indicates that, as P1 increased (as a result of orexin-A), there was a corresponding decrease in the early

inhibition ratio for population spike amplitude, meaning that there was more inhibition. Thus, larger P1 population spikes were associated with increased early inhibition, as expected.

For this reason input-output curve data taken initially and at the end of the experiments were used to compare changes in the paired pulse ratios when initial spike sizes were matched. See later sections 3.4.3.3 on page 38, 3.4.3.6 on page 40 and 3.4.3.9 on page 42.

3.4.3.2 Effect of Orexin-A on Control Group Paired Pulse Early Inhibition

There were no significant differences in paired pulse early inhibition pre and post injection for the control group. Mean early inhibition population spike amplitude paired pulse ratio for baseline was 0.29 (± 0.071) for the control group; the post orexin-A paired pulse ratio was 0.29 (± 0.084). EPSP slope paired pulse ratios for early inhibition are presented in Table 5; again there was no difference between this measure pre and post orexin-A.

Figure 6 shows the control group's P2/P1 early inhibition ratios for population spike amplitude. A repeated measures ANOVA revealed no significant differences between baseline and post orexin-A measures ($F_{11,33}=1.60$; ns). A repeated measures ANOVA for P2/P1 EPSP slope also showed no significant difference between baseline and post injection measures ($F_{11,33}=0.87$; ns).

Table 4 presents the results of a correlational analysis of P1 vs. P2/P1 early inhibition for population spike amplitude for the control group. Prior to injection P1 correlated

negatively with P2/P1 in two of 6 rats; after injection three of 6 rats showed a significant negative correlation (Table 4).

3.4.3.3 Effect of Orexin-A on Input-output Paired Pulse Early Inhibition Ratio

Figure 7 illustrates the early inhibition P2/P1 population spike amplitude ratios for the range of input-output currents prior to and following the orexin-A injection.

The early inhibition paired pulse ratio for population spike amplitude was compared using a t-test at 500 μ amp pre injection and at 250 μ amp post injection for the experimental group. The mean P2/P1 population spike ratio at 500 μ amp was 0.25 (± 0.13) prior to injection; following the orexin-A injection the mean P2/P1 population spike ratio at 250 μ amp was 0.16 (± 0.085). The difference between these means was not significant ($t_3=1.95$, ns). This result indicates that differences in the P2/P1 ratio may be accounted for by the increase in the P1 population spike amplitude.

The mean P2/P1 EPSP slope ratio for the experimental group at 500 μ amp was 0.86 (± 0.07); following the orexin-A injection the P2/P1 EPSP slope ratio at 250 μ amp was 0.87 (± 0.03). The difference between these means was also not significant ($t_3=0.15$, ns).

The early inhibition paired pulse ratio for population spike was compared using a t-test at 500 μ amp pre injection and at 500 μ amp post injection for the control group. as there was no change in population spike amplitude following the injection. The mean P2/P1 population spike ratio prior to orexin-A injection was 0.25 (± 0.08); after the injection the P2/P1 population spike ratio was 0.24 (± 0.09). There was no significant difference between the pre and post measures ($t_5=0.32$, ns).

The mean P2/P1 EPSP slope ratio for the control group before the injection was 0.78 (± 0.02); following the injection the P2/P1 EPSP slope ratio was 0.78 (± 0.02). The difference between these means was not significant ($t_5=0.06$, ns).

3.4.3.4 Effect of Orexin-A on Experimental Group Paired Pulse Facilitation

The mean facilitation population spike amplitude paired pulse ratio taken at 100 msec interstimulus interval for baseline was 2.12 (± 0.40) for the experimental group. Post orexin-A the facilitation population spike amplitude paired pulse ratio was reduced to 2.09 (± 0.32). The repeated measures ANOVA revealed that P2/P1 was significantly smaller post orexin-A than during the baseline period ($F_{11,33}=4.74$; $p<0.05$). Duncan post-hoc comparisons at $p=0.05$ showed that the facilitation P2/P1 population spike amplitude ratio was significantly smaller at 5 min post orexin-A and continued to decrease to about 2.00 at the end of the 30 min record (Figure 8). Table 5 provides the paired pulse ratios for EPSP slope. EPSP slope ratios during baseline and post orexin-A did not differ greatly.

Table 3 presents the P1 vs. P2/P1 correlation analysis for population spike in the experimental group. The P1 population spike amplitude correlated negatively with the facilitation P2/P1 ratio for all experimental animals. This analysis indicates that larger P1s were associated with less facilitation or possibly increased inhibition. However, this could be an artifact of a ceiling effect with larger P1s.

3.4.3.5 Effect of Orexin-A on Control Group Paired Pulse Facilitation

Mean facilitation population spike amplitude paired pulse ratio for baseline was 2.12 (± 0.21) for the control group; the post orexin-A paired pulse ratio was 2.25 (± 0.23).

There were no significant differences in this ratio after injection. EPSP slope paired pulse ratios for facilitation are presented in Table 5.

Table 4 presents the results of a correlational analysis of P1 vs. P2/P1 facilitation for population spike amplitude for the control group. Prior to injection all control animals had significant negative correlations for P1 vs. P2/P1 facilitation population spike amplitude; post injection 5 of 6 animals had significant negative correlations, albeit smaller correlations than pre measures.

3.4.3.6 Effect of Orexin-A on Input-output Paired Pulse Facilitation Ratio

Figure 9 illustrates the facilitation P2/P1 population spike amplitude ratios for the range of input-output currents prior to and following the orexin-A injection.

The facilitation paired pulse ratio for population spike amplitude was compared using a t-test at 500 μ amp pre injection and at 250 μ amp post injection for the experimental group. The mean P2/P1 population spike ratio at 500 μ amp was 1.97 (± 0.21) prior to injection; following the orexin-A injection the mean P2/P1 population spike ratio at 250 μ amp was 2.35 (± 0.33). The difference between these means was not significant ($t_3=1.077$, ns). This result indicates that differences in the facilitation P2/P1 population spike amplitude ratio may be accounted for by the increase in the P1 population spike amplitude.

The mean P2/P1 EPSP slope ratio for the experimental group at 500 μ amp was 0.96 (± 0.02); following the orexin-A injection the P2/P1 EPSP slope ratio at 250 μ amp was 0.97 (± 0.02). The difference between these means was also not significant ($t_3=1.008$, ns).

The facilitation paired pulse ratio for population spike was compared using a t-test at 500 μ amp pre injection and at 500 μ amp post injection for the control group. The mean P2/P1 population spike ratio prior to orexin-A injection was 1.68 (± 0.09); after the injection the P2/P1 population spike ratio was 1.80 (± 0.14). There was no significant difference between the pre post measures ($t_5=1.77$, ns).

The mean P2/P1 EPSP slope ratio for the control group before the injection was 0.93 (± 0.01); following the injection the P2/P1 EPSP slope ratio was 0.93 (± 0.01). The difference between these means was not significant ($t_5=0.88$, ns).

3.4.3.7 Effect of Orexin-A on Experimental Group Paired Pulse Late Inhibition

The mean late inhibition population spike amplitude paired pulse ratio at 500 msec interstimulus interval for baseline was 0.76 (± 0.02) for the experimental group. Post orexin-A the late inhibition population spike amplitude paired pulse ratio was reduced to 0.72 (± 0.042). The repeated measures ANOVA revealed that the P2/P1 was not significantly different post orexin-A than during the baseline period ($F_{1,33}=1.372$; ns); as is also seen in Figure 10. Table 5 illustrates the paired pulse ratios for EPSP slope. EPSP slope ratios during baseline and post orexin-A did not differ significantly.

Table 3 presents the P1 vs. P2/P1 correlation analysis for population spike for the experimental group. Prior to orexin-A, three of four rats showed a significant negative correlation between spike amplitude and late inhibition; following orexin-A all rats showed a significant negative correlation. This pattern suggests that as P1 increased there was a corresponding decrease in the late inhibition ratio for population spike amplitude, meaning that there was increased late inhibition.

3.4.3.8 Effect of Orexin-A on Control Group Paired Pulse Late Inhibition

Mean late inhibition population spike amplitude paired pulse ratio for baseline was 0.70 (± 0.07) for the control group; the post orexin-A paired pulse ratio was 0.62 (± 0.09). There were no significant differences in these ratios over time (Figure 10). EPSP slope paired pulse ratios for late inhibition are presented in Table 5; again there was no difference between this measure pre and post orexin-A injection.

Table 4 present the results of a correlational analysis of P1 vs. P2/P1 late inhibition for population spike amplitude for the control group. Prior to injection three of 6 control animals had significant negative correlations for P1 population spike amplitude vs. P2/P1 population spike amplitude ratio; post injection 5 of 6 animals had significant negative correlations.

3.4.3.9 Effect of Orexin-A on Input-output Paired Pulse Late Inhibition Ratio

Figure 11 illustrates the late inhibition P2/P1 population spike amplitude ratios for the range of input-output currents prior to and following the orexin-A injection.

The late inhibition paired pulse ratio for population spike amplitude was compared using a t-test at 500 μ amp pre injection and at 250 μ amp post injection for the experimental group. The mean P2/P1 population spike ratio at 500 μ amp was 0.92 (± 0.07) prior to injection; following the orexin-A injection the mean P2/P1 population spike ratio at 250 μ amp was 0.68 (± 0.04). The difference between these means was significant ($t_3=5.18$, $p<0.05$). This result indicates that there is an increase in the late inhibition P2/P1 population spike amplitude ratio at lower currents that cannot be accounted for by the increase in the P1 population spike amplitude.

The mean P2/P1 EPSP slope ratio for the experimental group at 500 μ amp was 0.93 (± 0.03); following the orexin-A injection the P2/P1 EPSP slope ratio at 250 μ amp was 0.89 (± 0.02). The difference between these means was not significant ($t_3=2.02$, ns).

The late inhibition paired pulse ratio for population spike was compared using a t-test at 500 μ amp pre injection and at 500 μ amp post injection for the control group. The mean P2/P1 population spike ratio prior to orexin-A injection was 0.79 (± 0.07); after the injection the P2/P1 population spike ratio was 0.78 (± 0.05). There was no significant difference between the pre post measures ($t_5=0.25$, ns).

The mean P2/P1 EPSP slope ratio for the control group before the injection was 0.91 (± 0.02); following the injection the P2/P1 EPSP slope ratio was 0.93 (± 0.01). The difference between these means was not significant ($t_5=2.37$, ns).

3.5 Frequency Dependent Inhibition

3.5.1 Effect of Orexin-A on Experimental Group Frequency Dependent Inhibition

Frequency dependent inhibition measures were collected approximately 35 minutes prior to orexin-A injection and 90 minutes following the injection. The mean 0.1 Hz population spike amplitude prior to the orexin-A injection for the experimental group was 3.08 mV (± 1.39); following the injection the mean 0.1 Hz population spike amplitude was 4.33 mV (± 1.97); $t_3=2.14$, ns. The mean EPSP slope for the experimental group was 4.09 mV/ms (± 1.57) before the orexin-A injection; following the injection the mean EPSP slope was 4.56 mV/ms (± 1.81); $t_3=1.74$; ns. Although not significant there was a 40% increase in population spike amplitude for the experimental group; in addition there

was also an 11% increase in EPSP slope. Both of these measures are consistent with a slowly developing potentiation.

The mean frequency dependent inhibition population spike amplitude ratio for the experimental group prior to orexin-A activation of the locus coeruleus was 0.65 (± 0.04); following the injection the mean frequency dependent inhibition population spike amplitude ratio was 0.66 (± 0.02). The mean frequency dependent inhibition EPSP slope ratio for the experimental group before orexin-A was 0.88 (± 0.01); after the orexin-A injection the EPSP slope ratio was 0.90 (± 0.04). There were no significant differences.

For the post baseline period, a matching procedure, in which the current was lowered to produce a 0.1 Hz population spike amplitude similar to baseline, was employed to control for changes in P1 after the orexin-A injection; matched values are reported for population spike and EPSP slope for the post period only. The mean matched frequency dependent inhibition population spike ratio for the experimental group was 0.72 (± 0.07); the mean matched frequency dependent inhibition EPSP slope ratio was 0.92 (± 0.04). There were no differences in the frequency dependent inhibition ratios pre and post injection for the matched data.

3.5.2 Effect of Orexin-A on Control Group Frequency Dependent Inhibition

The mean 0.1 Hz population spike amplitude for the control group prior to the orexin-A injection was 2.51 mV (± 0.42); following the injection the mean population spike amplitude was 2.48 mV (± 0.38); $t_5=0.19$, ns. The mean 0.1 Hz EPSP slope for the control group prior to the orexin-A injection was 3.15 mV/ms (± 0.35); the post injection mean EPSP slope was 3.06 mV/ms (± 0.33); $t_5=0.19$, ns.

The mean frequency dependent inhibition population spike amplitude ratio for the control group prior to the orexin-A injection was 0.61 (± 0.10). Following the injection the frequency dependent inhibition ratio for population spike amplitude was 0.55 (± 0.07). The mean post injection matched frequency dependent inhibition population spike amplitude ratio was 0.56 (± 0.03); the mean frequency dependent inhibition EPSP slope for the control group before the orexin-A injection was 0.93 (± 0.02); the mean frequency dependent inhibition ratio for the period following the injection was 0.94 (± 0.01). The pre and post injection frequency dependent inhibition ratios for spike amplitude and EPSP slope did not differ. The difference between these two means was not significant ($t_5=0.86$; ns).

3.6 Experimental Versus Control Group Differences

Experimental and control group population spike amplitudes, EPSP slopes and the various paired pulse measures were examined using two-way ANOVA. Prior to orexin-A injection there was no significant difference between the experimental and control groups. The experimental group was significantly different from the control group for population spike amplitude at all time points following injection of orexin-A ($F_{1,9}=5.86$; $p<0.05$). There were no differences between the experimental and control groups on EPSP slope or any of the paired pulse measures. Using the matched post orexin-A frequency dependent inhibition population spike amplitude the experimental group had a significantly larger ratio (0.72 vs. 0.56) than the control group ($t_9=2.37$; $p<0.05$); this may reflect an increase in feed forward inhibition in the experimental animals compared to the control group. The groups were not significantly different prior to the orexin-A injection.

The frequency dependent inhibition EPSP slope did not differ between the groups following orexin-A infusion.

Differences between the experimental and control group were compared using the paired pulse ratios for the input output measures. While there was a trend for the late inhibition paired pulse ratio measures to be lower in the experimental animals following orexin-A, the difference did not reach statistical significance, possibly because of the small sample size of the experimental group. The groups did not differ on the measures of early inhibition or facilitation, either before or after orexin-A injection.

3.7 Summary

Orexin-A infusion into the locus coeruleus produced a 122% increase over baseline in population spike amplitude in the experimental group; larger population spikes tended to occur with smaller EPSPs. There were no differences in the control group. Late paired pulse inhibition was significantly altered in the experimental group, with both population spike amplitude and late inhibition P2/P1 population spike amplitude ratio being reduced following the orexin-A injection. While there were no detectable differences in frequency dependent inhibition in the experimental group following orexin-A injection as compared to pre-injection, this measure did indicate greater feed-forward inhibition in the experimental group following orexin-A injection as compared to the control group.

4. Discussion

The present experiments were directed at analyzing the effects of orexin-A on three types of recurrent phenomena found with perforant path stimulation in that circuitry: (1) early inhibition, (2) facilitation and (3) late inhibition. The results replicated a previous report that orexin-A injection in or near the locus coeruleus results in a gradually developing, significant enhancement of EPSP slope and population spike amplitude (Walling et al., 2004). The present study also demonstrated for the first time a possible noradrenergic enhancement of late inhibition. Contrary to earlier claims (e.g., Sara and Bergis, 1991), there was no modulation of early inhibition that could not be accounted for by the indirect effects of an enhanced population spike. A similar result was observed for facilitation. Frequency-dependent inhibition, which was probed as a measure of NMDA receptor modulation, displayed mixed results. The within group measure suggested no change in frequency dependent inhibition, while the between group measure suggested frequency dependent inhibition was reduced. Each of these results will be considered in turn.

4.1 Orexin-A Infusion in the Locus Coeruleus Replicates Noradrenergic Effects on the First Perforant Path Evoked Potential

In the present study orexin-A increased the perforant path population spike to 122% of baseline at 30 minutes post-infusion for the experimental group. The EPSP slope increased to 108% of baseline over the same period for the experimental group. These changes in response to the baseline perforant path current mediated by locus coeruleus activation by orexin-A were confirmed in the input-output curves compared before, and

after, baseline recording in which larger population spikes are seen following infusion at all current levels that produce spikes. Average slope in the input-output curves was also elevated. Control population spike amplitude and EPSP slope values were unchanged by using either baseline or input-output measures. Using between-group analyses, only the population spike amplitude following orexin-A infusion was significantly different between experimental and control groups indicating the robustness of the spike enhancement.

Potentiation of the perforant path population spike is consistently reported with the application of norepinephrine *in vitro* (Lacaille and Harley, 1985; Stanton and Sarvey, 1985), iontophoretically (Neuman and Harley, 1983) or through glutamatergic (Harley and Milway, 1986) or electrical (Babstock and Harley, 1992; Harley et al., 1989) activation of the locus coeruleus. Potentiation effects of 130-400% of baseline have been reported in these studies.

In a previous study of orexin-A infusion in the locus coeruleus (Walling et al., 2004) a similar population spike amplitude increase to the one observed here was observed at 30 minutes, however, with a longer recording period potentiation continued to develop to 155% of baseline at 3 hr post injection. A prolonged development of population spike potentiation appears to characterize orexin-A locus coeruleus activation effects on the perforant path potential and distinguishes it from the profile observed with glutamate activation of the locus coeruleus. With glutamate activation, spike potentiation reaches asymptote more rapidly and may decline from an initially higher level (Harley and Sara, 1992; Klukowski and Harley, 1994).

This difference in the development of population spike potentiation could relate to the profile of locus coeruleus unit firing induced by the two methods. With orexin-A infusions there is a steady increase in tonic firing for a limited period on the order of 20 minutes or less (Walling et al., 2004), while with glutamate there is a brief intense barrage of firing lasting less than 500 ms that is followed by inhibition of locus coeruleus firing over minutes before baseline activity resumes (Harley and Sara, 1992). Both types of activation produce a similar elevation of norepinephrine in the hippocampus (~200%) for the first microdialysis sample (20 minutes) with a return to basal norepinephrine by the second sample (40 minutes later) (Walling et al., 2004).

Thus, effects on hippocampal neurophysiology with either glutamatergic or orexinergic activation that are seen beyond 20 minutes are not related to the continuing direct action of norepinephrine, but reflect norepinephrine-initiated changes in cell responses. During the initial 20 minutes it is likely that norepinephrine release occurs in a different pattern with glutamatergic and orexinergic activation, but microdialysis cannot resolve these temporal differences.

Increases in EPSP slope have been variably reported with locus coeruleus activation (Babstock and Harley, 1992; Harley and Milway, 1986), although they are more consistently observed in the *in vitro* studies (Stanton and Sarvey, 1985). In the previous study of orexin-A effects (Walling et al. 2004) an early transient EPSP slope increase was reported, but no enduring changes were observed. Here the EPSP slope does show lasting change. Thus, orexinergic locus coeruleus activation also appears to have variable effects on EPSP slope *in vivo*.

The increase in EPSP slope does not account for the increase in population spike supporting the earlier characterization of noradrenergic effects *in vivo* (Munro et al., 2001; Richter-Levin et al., 1991) and *in vitro* (Robinson and Racine, 1985; Sarvey et al., 1989) as increases in the coupling of EPSP slope to spike generation or as an increase in cell excitability.

4.2 Modulation of the Second Perforant Path Evoked Potential Population Spike by Orexin-A Infusion into the Locus Coeruleus Varies Depending on the Paired Pulse Interval

If the second population spike in each paired pulse is considered alone, the second spikes from short interval pairs decrease after orexin-A activation of the locus coeruleus, whereas the second spikes at longer intervals tended to increase in amplitude. This pattern suggests the increased driving of the first population spike promotes greater feedback inhibition at short intervals despite any general potentiating effects of norepinephrine release. There were no significant changes in EPSP slope for any of the second perforant path evoked potentials in a pair, although the trend was for increases.

The second population spikes at the intermediate facilitation interstimulus interval showed increases, but these increases were not significant. Second population spikes at the longer late inhibition interval did significantly increase over time in a degree similar to that of the first population spike potentiation. This could be due to general noradrenergic potentiation effects operating on the second population spike as well as on the first population spike in a pair or to a reduction in late inhibition. As discussed below a reduction in late inhibition does not appear to occur, but rather the converse.

4.3 Spike Matching Significantly Alters Apparent Changes in Evoked Potentials at Each of the Three Paired Pulse Intervals, but does not Affect Frequency-Dependent Inhibition

Without spike matching early feedback inhibition mediated by GABA-A receptors (DiScenna and Teyler, 1994) appeared to increase after locus coeruleus activation by orexin-A as the ratio of the second population spike to the first was significantly smaller in the last 15 minutes post orexin-A. However, since all experimental subjects and half of the control subjects demonstrated a significant negative correlation between size of the first population spike and the P2/P1 ratio for early inhibition in the baseline period, an increase in that ratio with an increase in P1 is expected.

When the input-output curve relating the early inhibition ratio to current was used to find currents that gave matching P1 population spike amplitudes for the experimental group, there was no change in the P2/P1 ratio. Thus, early inhibition, independent of P1 increases, was unchanged in the present study. This contrasts with the conclusion reached in an earlier test of feedback inhibition change with norepinephrine elevation, in which the alpha 2 antagonist, idazoxan, was used to elevate norepinephrine (Sara and Bergis, 1991). In this study greater feedback inhibition was reported, but only at a 25 ms interstimulus interval when inhibition decreased from a ratio of just over 0.6 to just under 0.4 after norepinephrine elevation. At other interstimulus intervals, including both shorter intervals with more inhibition, as in the present study, and longer intervals with similar or less feedback inhibition, no change in inhibition was observed. The authors

suggest norepinephrine produced an increase in feedback inhibition unrelated to an increase in the first spike because in one rat the first spike did not change while the increased feedback inhibition at 25 ms interstimulus interval was still observed. Thus the authors argued for independent effects of norepinephrine on population spike potentiation and feedback inhibition.

The present data are at odds with this conjecture. However, in the present study, the interstimulus interval used was 5 ms shorter than that which gave increased feedback inhibition in the Sara and Bergis study. The 20 ms interstimulus interval in the Sara and Bergis study produced a P2/P1 ratio of approximately 0.3 and was not changed after idazoxan administration.

The difference in interstimulus interval may account for the different outcome between studies. The Sara and Bergis study also differed in other ways. Rats were unanesthetized in the Sara and Bergis study. There was no control for the size of the first spike in 7 of 8 rats and antagonism of alpha 2 receptors is a different mechanism of norepinephrine increase that may alter the effect profile. Idazoxan also affects imidazoline receptors (Hudson et al., 1999; Mallard et al., 1992), which could contribute to differences in outcomes.

It is surprising that Sara and Bergis did not observe a difference in feedback inhibition at any interval other than 25 ms since the present data in both control and experimental rats strongly suggest that larger population spikes should recruit increased levels of early feedback inhibition. They had larger spikes at all intervals but increased inhibition at only one interval. If input/output curves had been taken and the normal

spike relationship to early feedback inhibition obtained, it might even be argued that their data show feedback inhibition at other intervals is reduced.

Small sample size is a concern in the present study. The feedback ratio with matched population spikes was smaller after orexin A (0.16 versus 0.25), but this difference, with an N of four, was not significant. Thus, more subjects would be useful before drawing a final conclusion about early feedback inhibition.

At intermediate paired pulse intervals, the normal population spike facilitation appeared to decrease with a smaller ratio being observed after orexin-A activation of locus coeruleus. For the facilitation ratio, population spike amplitude was negatively correlated with the facilitation ratio in experimental and control rats. So, again, to assess whether there is a real change in facilitation, it was necessary to control population spike size using input-output curve data for the facilitation ratio. There were no significant differences in population spike or EPSP slope facilitation ratios when amplitude of the first population spike was matched.

At the longest paired pulse interval, late inhibition ratios did not change significantly after orexin-A infusion in the locus coeruleus. However, late inhibition ratios during baseline were also negatively correlated with spike size with larger spikes associated with greater late inhibition. Thus, even though this relationship was not evident in the post-orexin paired pulse baseline data, late inhibition input-output ratios were examined under conditions that matched the first population spike amplitude. In this case, when spikes were matched in amplitude, there was a clear and significant increase in late inhibition following orexin-A activation of the locus coeruleus. The late

inhibition ratio was approximately 0.9, thirty minutes prior to orexin-A infusion into the locus coeruleus, while the same size P1 produced a late inhibition ratio of less than 0.7, 30 minutes after orexin-A infusion. These effects are clearest with small spikes that may reflect a more physiological level of granule cell activation. This pattern of results suggests a long-lived increase in the GABA-B mediated effects that underlie late inhibition (Davies and Collingridge 1993; Mott and Lewis, 1994; Stanford et al., 1995) after norepinephrine release in the dentate gyrus.

Frequency-dependent inhibition is not altered when examined 30 minutes after orexin-A infusion into the locus coeruleus. When population spike matching was used for the pre and post frequency-dependent inhibition comparisons by adjusting stimulation current to approximate the spike size prior to orexin-A infusion, there was still no difference in frequency-dependent inhibition within the experimental group. With matching, the frequency-dependent inhibition for experimental rats after infusion of orexin-A was less than that seen in controls, but this may be a sampling error, since the more sensitive within-test did not detect a difference.

Frequency-dependent inhibition is influenced both by GABA-A activation (Maroun and Richter-Levin, 2002; Sloviter, 1991) and by NMDA receptor activation (Rosenblum et al., 1999), so it is not surprising that this measure does not change. In the context of the present study GABA-A function appears unaltered by norepinephrine release in early feedback inhibition and there is no previous evidence for long-lasting changes in NMDA receptor function with norepinephrine release. Some evidence does suggest that there are direct norepinephrine effects on NMDA receptors (Burgard et al.,

1989; Harley, 1991; Stanton et al., 1989), but not long-lasting ones. To test a direct norepinephrine influence on NMDA action, it would be necessary to examine frequency-dependent inhibition immediately after locus coeruleus activation when norepinephrine is likely to be present in the target area. The lack of change in the facilitation ratio with matched spikes, a measure also taken after 30 minutes, also suggests no enduring effect on NMDA receptor function. NMDA receptor enhancement has been suggested to underlie paired pulse facilitation (Joy and Albertson, 1993), although there is also evidence for other mechanisms (Andreasen and Hablitz, 1994; Brucato et al., 1995).

While the frequency dependent inhibition measure, and those measures that required the input-output curves to equate for spike amplitude were taken 30 minutes after locus coeruleus activation by orexin-A and are, presumably, outside the window of direct norepinephrine influence, the first evoked potential and the other paired pulse measures were taken continually from the point of orexin-A infusion into the locus coeruleus. There were no changes that were confined to the likely period of direct norepinephrine influence. The most common pattern was a gradual increase in effect strength with time after locus coeruleus activation suggesting most effects were initiated by, but not continually dependent on, norepinephrine.

4.4 Conclusions

In summary, an increase in GABA-B mediated late inhibition appears to be a second enduring effect of locus coeruleus activation in the dentate gyrus with the first enduring effect being that of potentiation of the population spike amplitude. As far as is known, this is the first report of enduring change in an inhibitory circuit in the dentate

gyrus other than in pathological conditions. Increased GABA-B inhibition might account for norepinephrine's ability to reduce epileptic seizures in a variety of models (Kamphuis et al., 1991). It would also tend to exaggerate the theta rhythm, since inhibition occurring ~200 ms after principal cell activation would be enhanced. Such an exaggeration of excitatory/inhibitory cycles could contribute to the increase in theta power reported to occur with locus coeruleus activation (Berridge et al., 1993; Berridge and Foote, 1991).

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6. Tables

Table 1: Experimental group EPSP slope versus population spike correlations

Subject	Slope vs population spike pre orexin-A	Sign.	Slope vs population spike post orexin-A	Sign.
16	0.037	ns	-0.261	*
19	0.093	ns	-0.355	**
21	0.374	**	0.578	***
27	0.457	***	0.530	***

Table 2: Control group EPSP slope versus population spike correlations

Subject	Slope vs population spike pre orexin-A	Sign.	Slope vs population spike post orexin-A	Sign.
11	0.499	***	-0.031	ns
12	0.326	*	0.378	**
13	0.072	ns	-0.211	ns
14	0.058	ns	0.034	ns
18	-0.014	ns	-0.0354	ns
22	0.659	***	0.2374	ns

Table 3: Experimental group P1 versus P2/P1 population spike correlations

Subject	EI pre	Sign.	EI post	Sign.	FAC pre	Sign.	FAC post	Sign.	LI pre	Sign.	LI post	Sign.
16	-0.849	***	-0.850	***	-0.956	***	-0.954	***	-0.580	***	-0.642	***
19	-0.348	ns	-0.864	***	-0.842	***	-0.916	***	-0.661	***	-0.614	***
21	-0.649	***	-0.675	***	-0.926	***	-0.856	***	-0.751	***	-0.536	**
27	0.174	ns	-0.542	**	-0.849	***	-0.634	***	-0.234	ns	-0.594	***

Table 4: Control group P1 versus P2/P1 population spike correlations

Subject	EI pre	Sign.	EI post	Sign.	FAC pre	Sign.	FAC post	Sign.	LI pre	Sign.	LI post	Sign.
11	-0.322	ns	-0.559	**	-0.586	***	-0.399	*	-0.229	ns	-0.690	***
12	-0.781	***	-0.681	***	-0.944	***	-0.962	***	-0.304	ns	-0.698	***
13	0.191	ns	-0.140	ns	-0.760	***	-0.127	ns	-0.793	***	-0.827	**
14	-0.075	ns	-0.202	ns	-0.825	***	-0.754	***	-0.761	***	-0.274	ns
18	-0.050	ns	-0.052	ns	-0.888	***	-0.883	***	-0.584	***	-0.645	***
22	-0.530	**	-0.552	**	-0.900	***	-0.522	**	-0.088	ns	-0.664	***

Table 5: Average P1 EPSP slope and EPSP slope paired pulse ratios for experimental and control groups

Condition	P1 (mV/ms) Pre	P1 (mV/ms) Post	P2/P1 EI Pre	P2/P1 EI Post	P2/P1 FAC Pre	P2/P1 FAC Post	P2/P1 LI Pre	P2/P1 LI Post
Experimental	4.193 (± 1.621)	4.302 (± 1.687)	0.914 (± 0.040)	0.894 (± 0.025)	0.955 (± 0.016)	0.961 (± 0.020)	0.913 (± 0.023)	0.922 (± 0.024)
Control	3.271 (± 0.364)	3.20 (± 0.368)	0.824 (± 0.018)	0.821 (± 0.019)	0.920 (± 0.013)	0.908 (± 0.014)	0.913 (± 0.014)	0.921 (± 0.014)

Table 6: Average P1 population spike and population spike paired pulse ratios for experimental and control group

Condition	P1 (mV) Pre	P1 (mV) Post	P2/P1 EI Pre	P2/P1 EI Post	P2/P1 FAC Pre	P2/P1 FAC Post	P2/P1 LI Pre	P2/P1 LI Post
Experimental	3.059 (± 1.427)	3.729 (± 1.772)	0.284 (± 0.127)	0.232 (± 0.095)	2.119 (± 0.401)	2.091 (± 0.317)	0.7631 (± 0.018)	0.720 (± 0.042)
Control	2.576 (± 0.374)	2.531 (± 0.384)	0.286 (± 0.071)	0.293 (± 0.084)	2.116 (± 0.214)	2.251 (± 0.232)	0.698 (± 0.069)	0.617 (± 0.089)

7. Figures

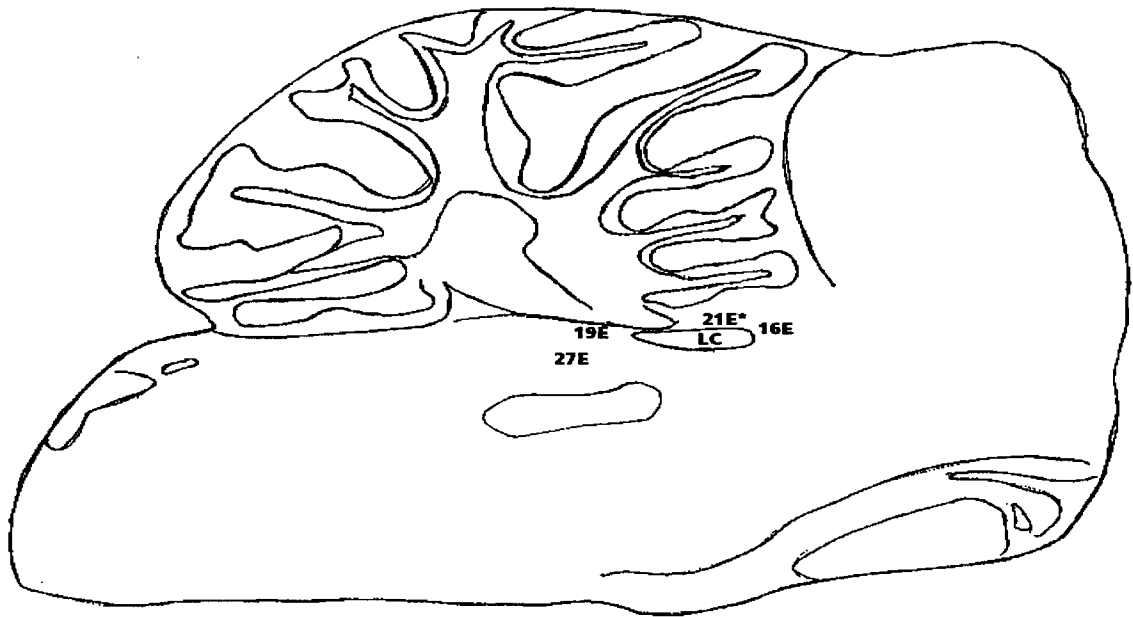


Figure 1a. Locus coeruleus electrode placements for the experimental group. 21E placement was approximately 160 μm lateral to the locus coeruleus (LC).

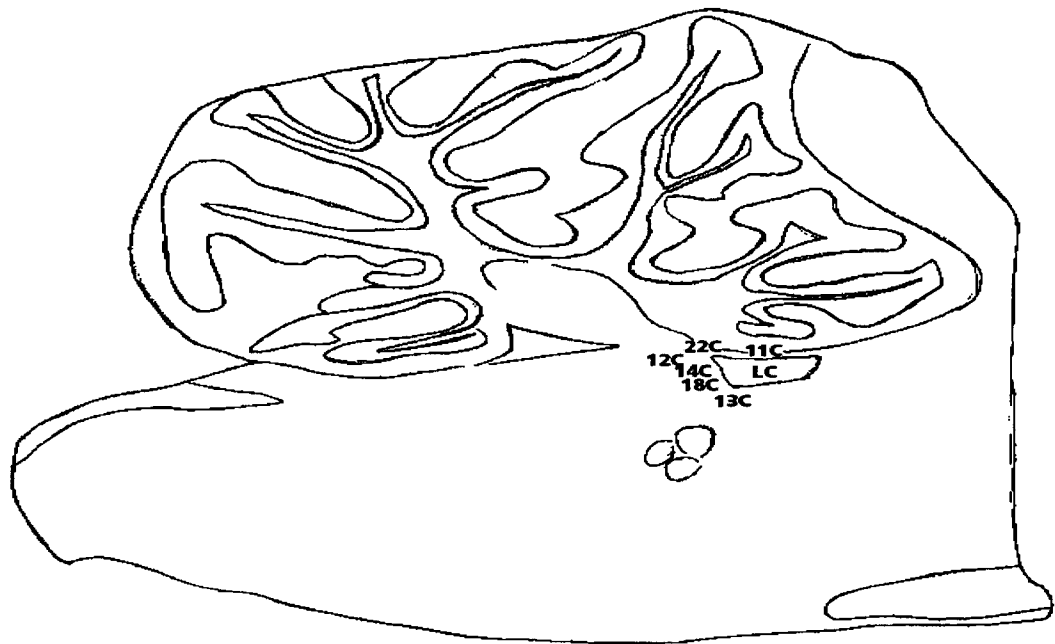


Figure 1b. Locus coeruleus electrode placements for the control group. 11C, 12C, 13C and 14C received inactive forms of orexin-A. 18C and 22C had placements that were between 360 and 520 μm lateral to the locus coeruleus (LC).

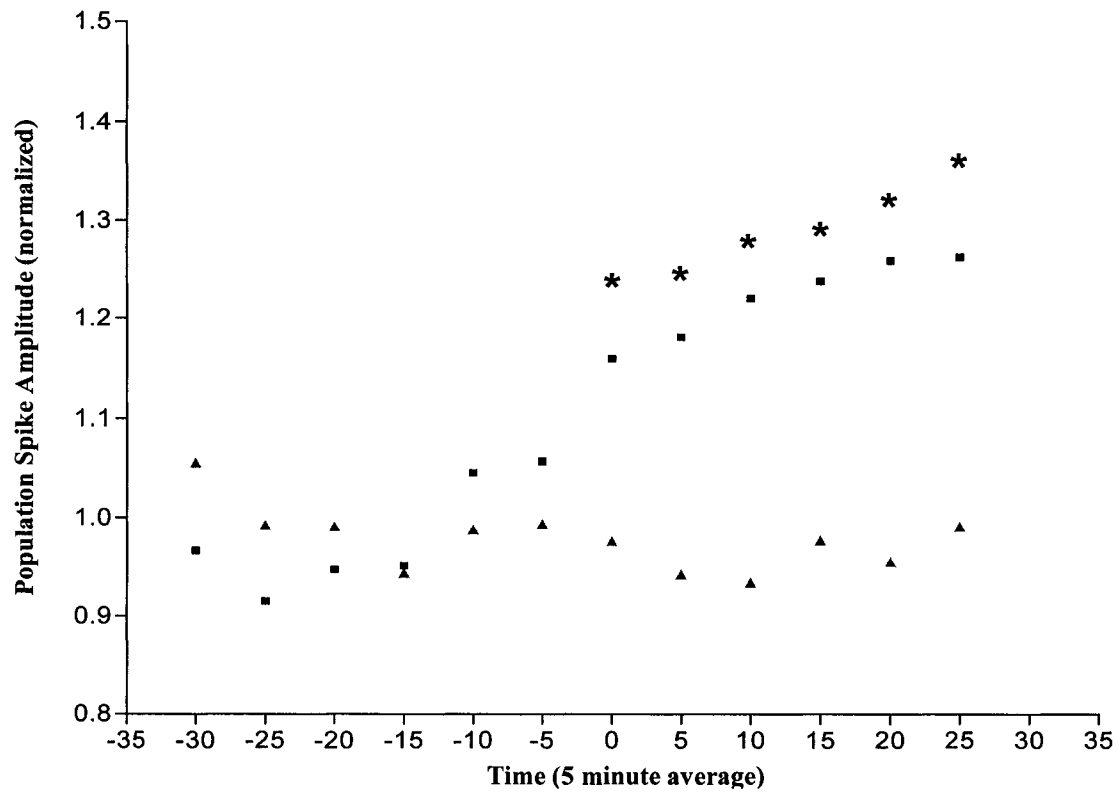


Figure 2: P1 population spike amplitude for experimental (■) and control (▲) groups. P1 population spike amplitude increased at the point of orexin-A injection and continued to increase over the 30 minute post injection period for the experimental group.

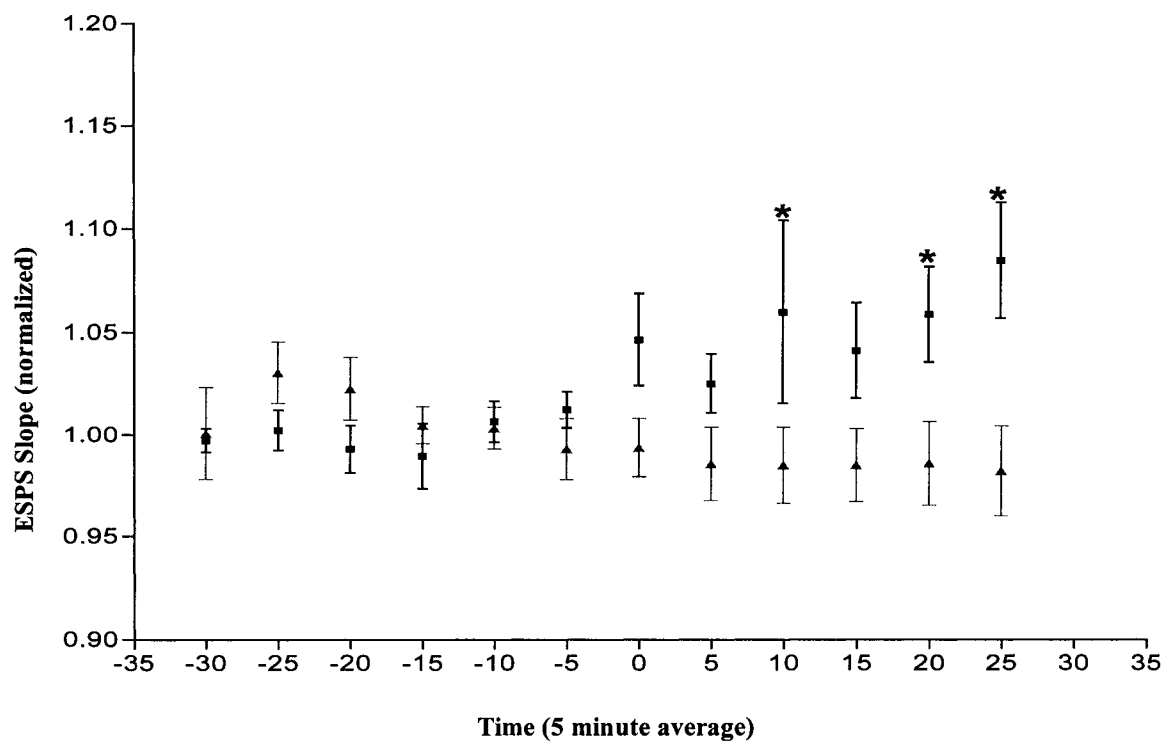


Figure 3: P1 EPSP slope for experimental (■) and control (▲) groups. P1 EPSP slope was significantly greater than baseline at 10 minutes post orexin-A and consistently larger from 20 minutes out, until the end of the post injection period for the experimental group.

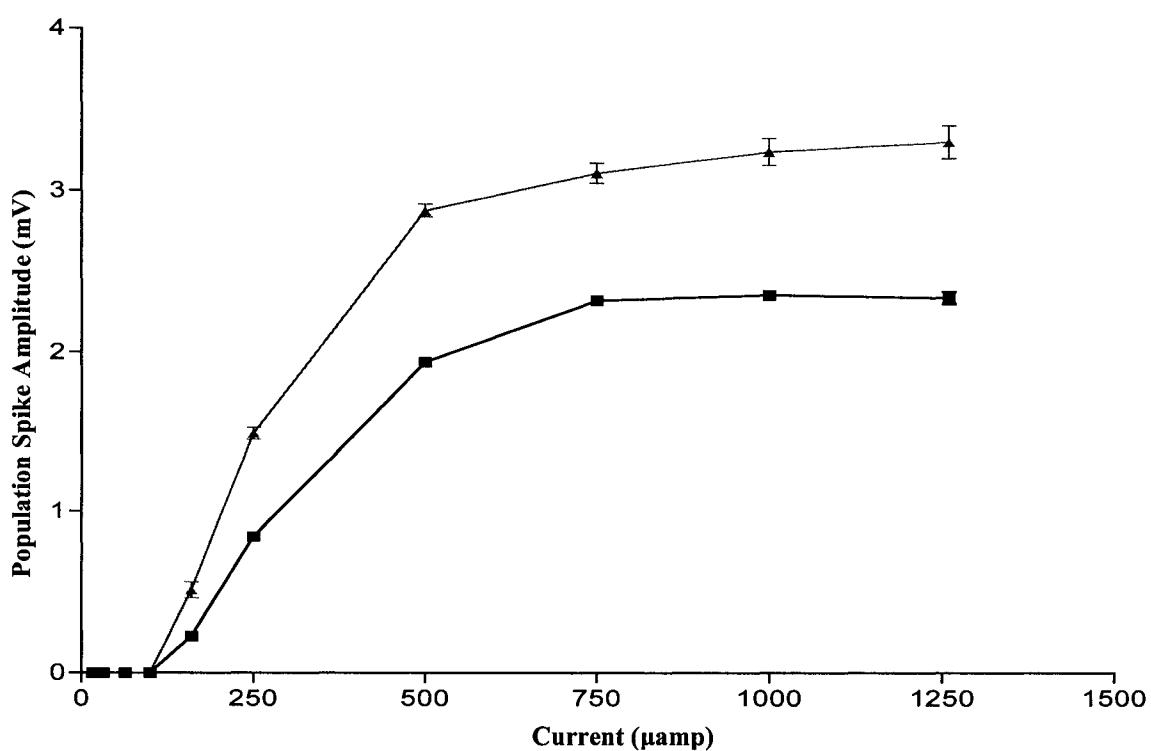


Figure 4: All pre (■) vs. post (▲) P1 population spike amplitudes for the experimental group. At all currents with a measurable population spike, population spike amplitudes are larger following orexin-A activation of the norepinephrine system than before this activation.

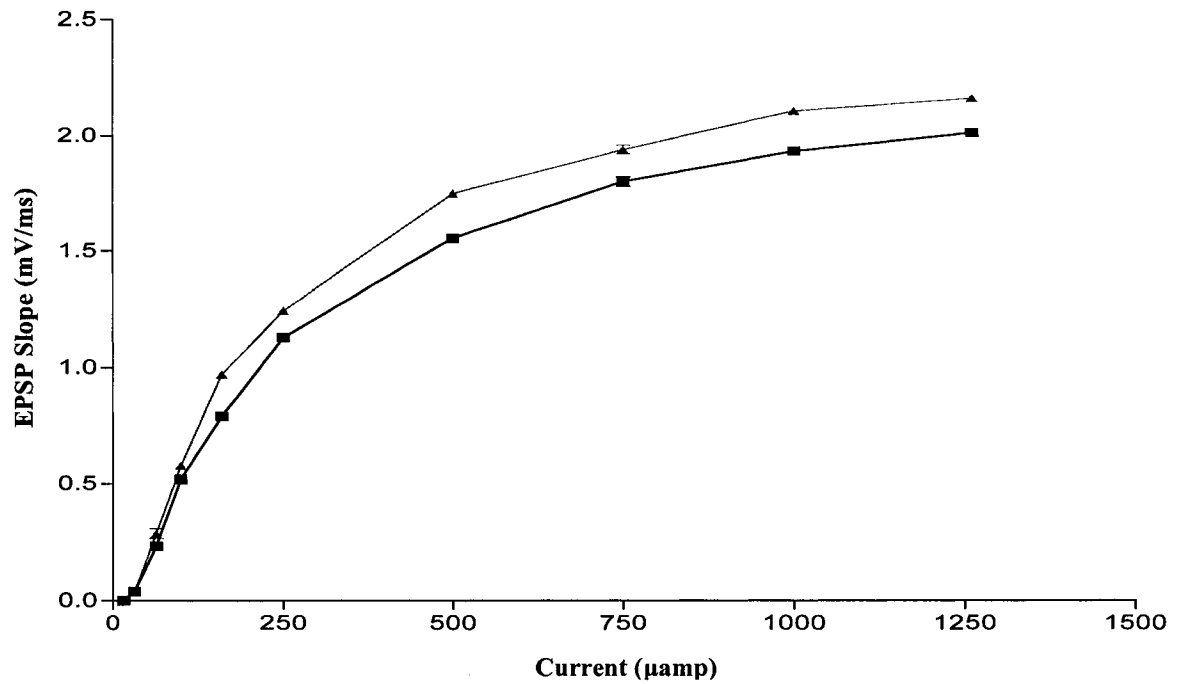


Figure 5: All pre (■) vs. post (▲) EPSP slope for experimental group. P1 EPSP slopes are larger following the orexin-A injection; however, they are not of the magnitude of the increase in P1 population spike.

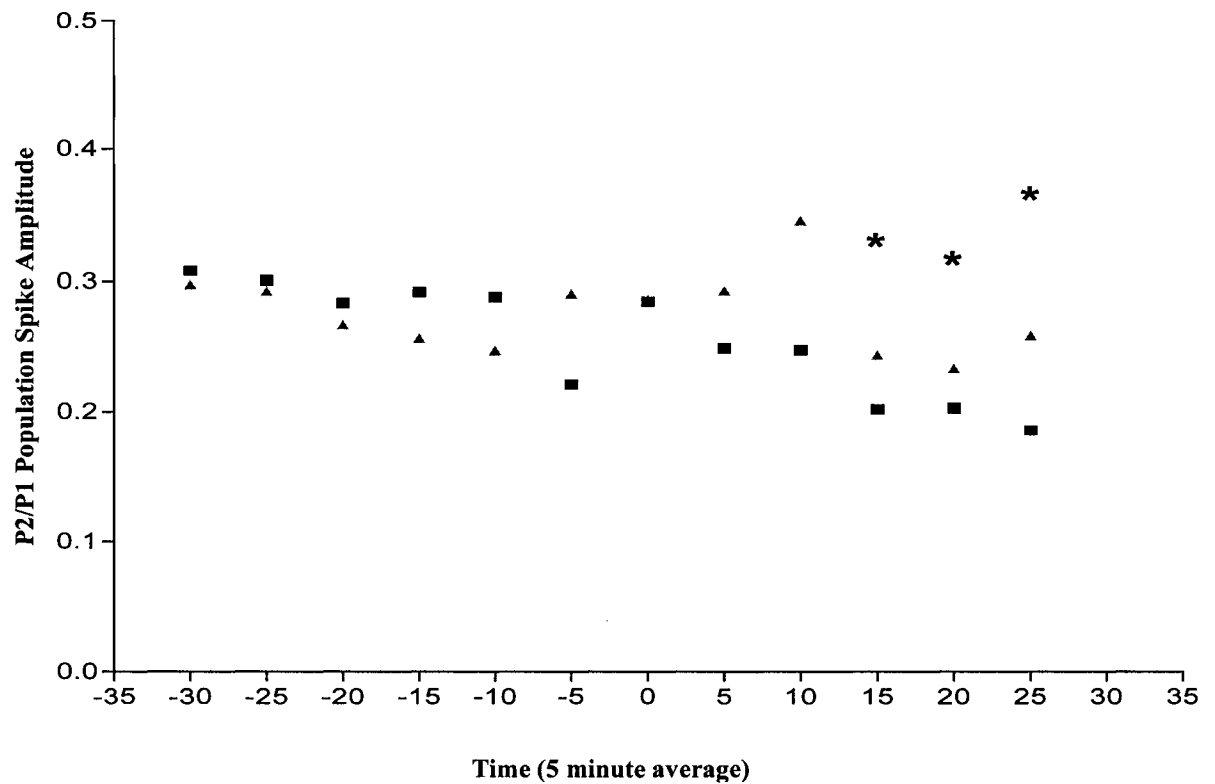


Figure 6: Early inhibition P2/P1 population spike amplitude ratio for experimental (■) and control (▲) groups. Early inhibition P2/P1 population spike amplitude ratio was significantly smaller at 15 minutes post orexin-A and continued to decrease to the end of the 30 minute record.

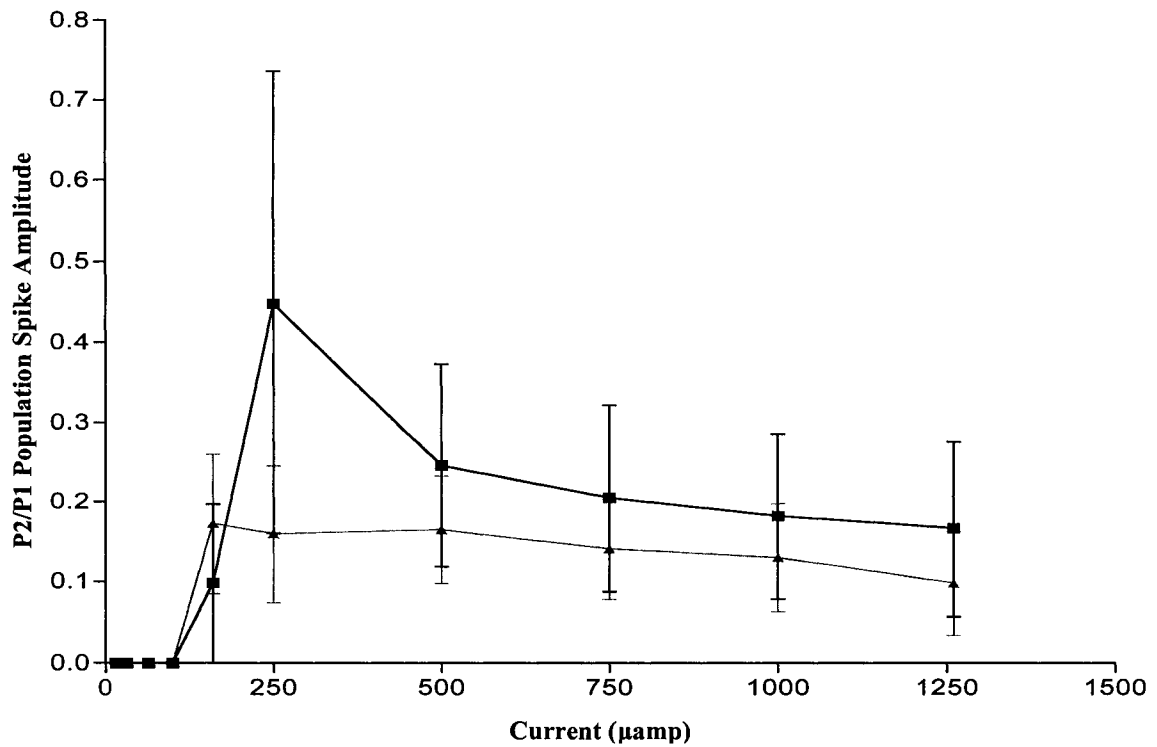


Figure 7: Early inhibition P2/P1 population spike amplitude ratio for input-output curves pre (■) and post (▲) orexin-A infusion for experimental group. Pre and post early inhibition measures did not differ, which indicates that differences in the P2/P1 ratio may be accounted for by the increase in the P1 population spike Amplitude.

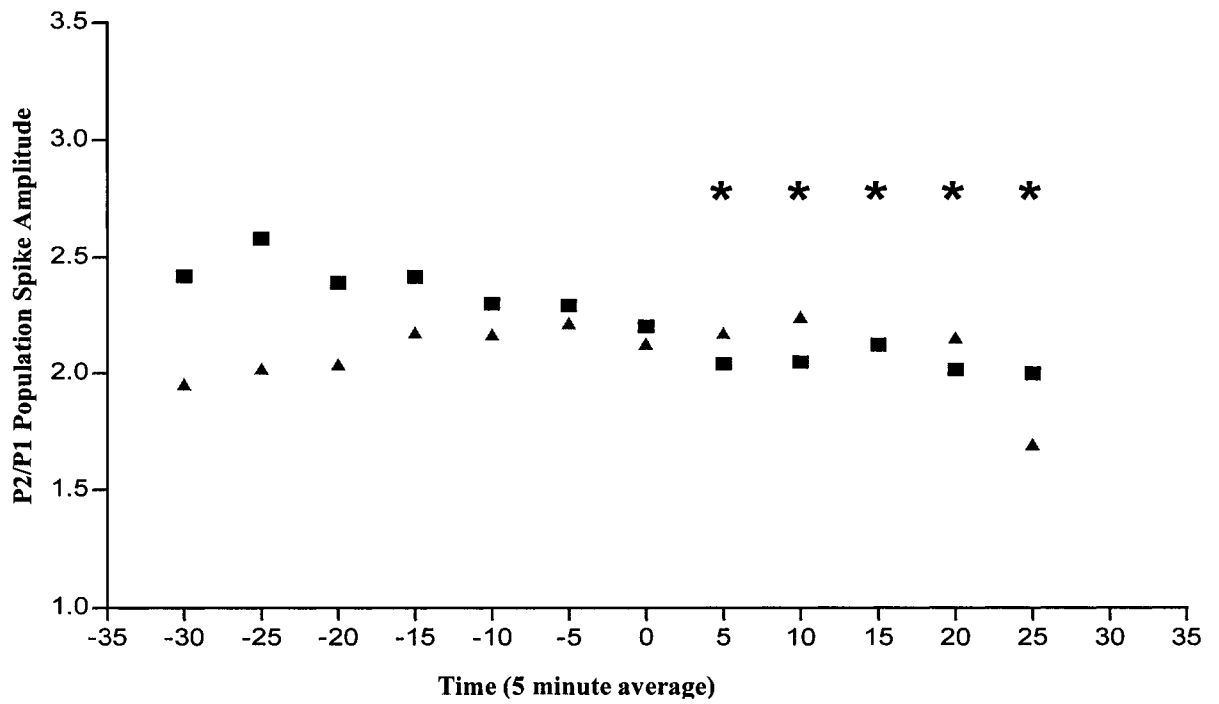


Figure 8: Facilitation P2/P1 population spike amplitude ratio for experimental (■) and control (▲) groups. The facilitation P2/P1 population spike amplitude ratio was significantly smaller at 5 minutes post orexin-A and continued to decrease to the end of the 30 minute record.

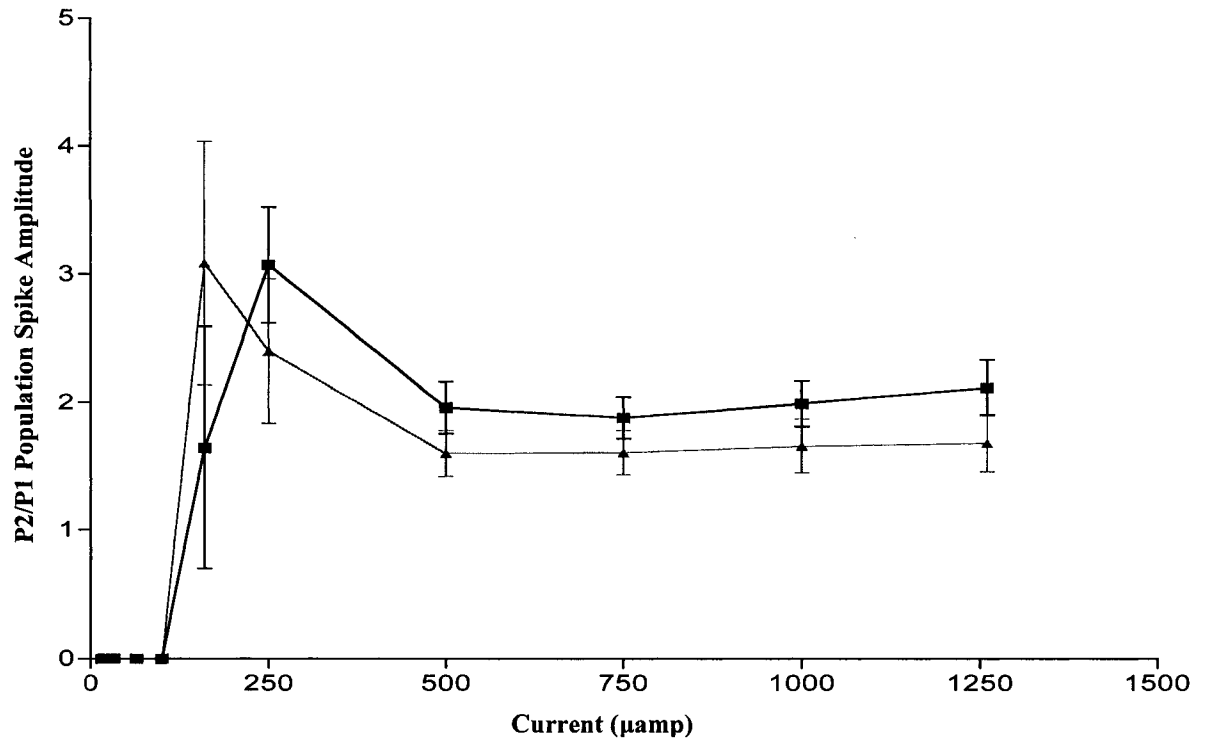


Figure 9: Facilitation P2/P1 population spike amplitude ratio for input-output curves pre (■) and post (▲) orexin-A infusion for experimental group. Pre and post facilitation measures did not differ, which indicates that differences in the P2/P1 ratio may be accounted for by the increase in the P1 population spike amplitude.

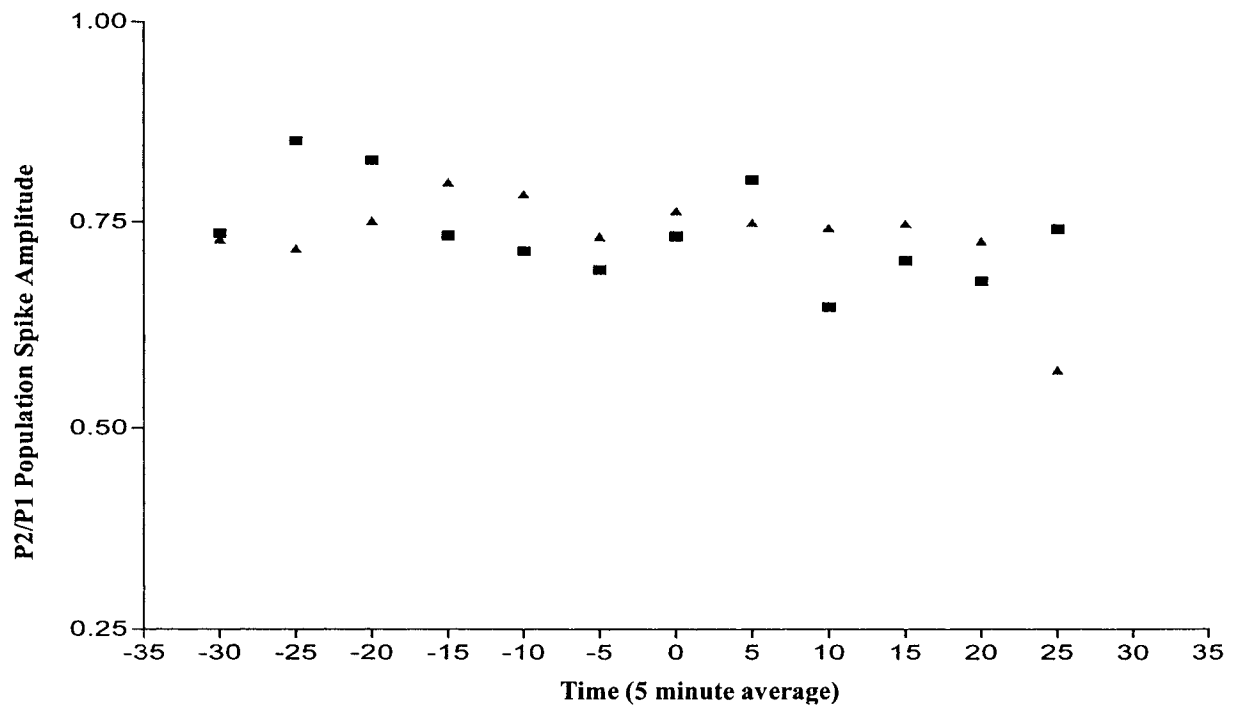


Figure 10: Late inhibition P2/P1 population spike amplitude ratio for experimental (■) and control (▲) groups. The late inhibition P2/P1 population spike amplitude ratio did not differ pre and post orexin-A infusion.

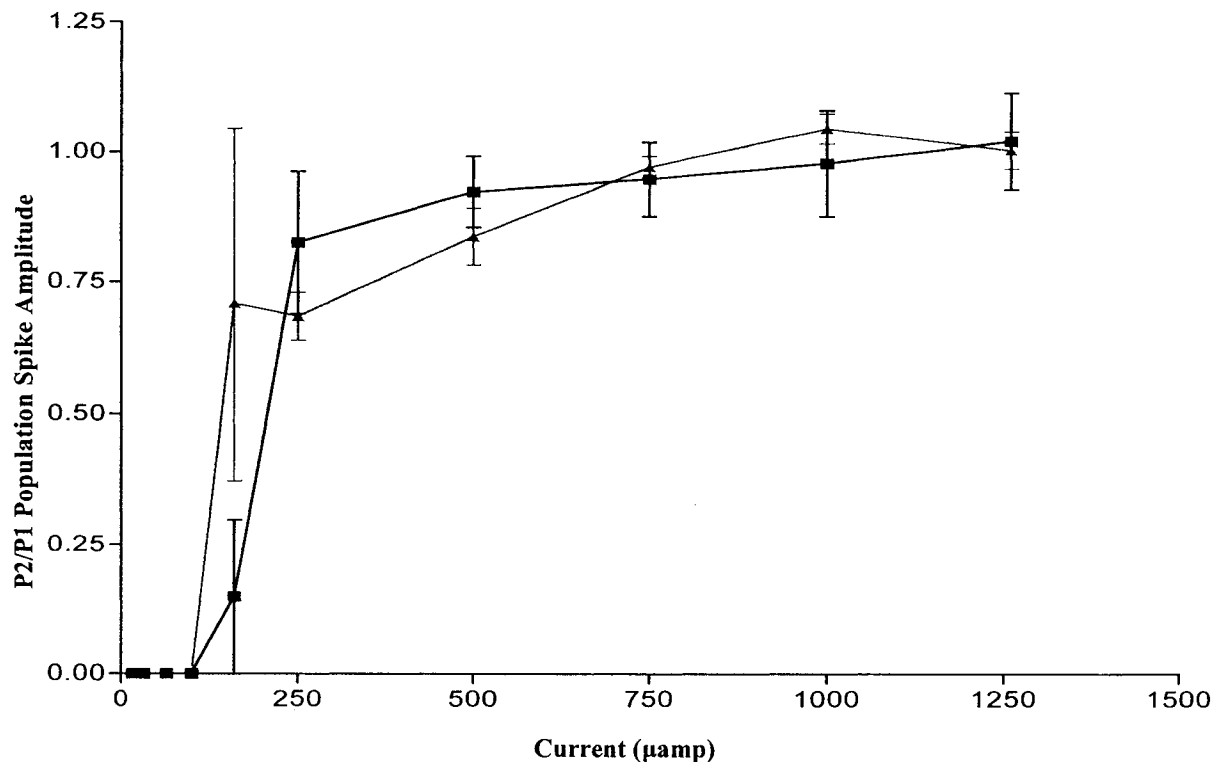


Figure 11: Late inhibition P2/P1 population spike amplitude ratio for input-output curves pre (■) and post (▲) orexin-A infusion for experimental group. Pre and post late inhibition measures did differ, which indicates that there is an increase in the late inhibition P2/P1 population spike amplitude ratio at lower currents that cannot be accounted for by the increase in the P1 population spike amplitude.



